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14. ABSTRACT

Here, we report that Tumor-associated macrophages (TAMs) promote Cancer stem cell (CSC) -like phenotypes in murine breast cancer cells by up regulating their expression of Sox-2, resistance to chemotherapy, and increased tumorigenicity. Down regulation of Sox-2 in tumors blocked the ability of TAMs to induce these CSC-like phenotypes and inhibited tumor growth. We identified a novel EGFR/ Stat3/Sox-2 paracrine signaling pathway between macrophages and breast cancer cells and showed that this crosstalk was effectively blocked by small molecule inhibitors AG1478 or CDDO-Im against EGFR and Stat3, respectively. Therefore, our study identifies a novel role for TAMs in breast CSC regulation and establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for CSC therapy.

Intratumoral injection of miR-19a-3p impaired the capacity of breast tumor cells to migrate and invade, suggesting it to play a critical role in induction of macrophage polarization and to be a useful therapeutic target for remodeling the tumor immune environment and thereby improve treatment of breast cancer.

15. SUBJECT TERMS
Sox-2 upregulates CSC phenotype; TAMs mediates tumorigenesis; Sox-2 signaling is controlled by Stat-3; EGRF/Sox-2 signlaing is a therapy target

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4. INTRODUCTION:

The cancer stem cell CSCs) hypothesis postulates that neoplastic clones are maintained by a small subpopulation of cells that possess the capacity for self-renewal and differentiation. CSCs were been suggested to be the root cause of cancer recurrence and disease relapse due to their resistance to chemo- and radiotherapy. CSCs were first identified in hematologic tumors and were subsequently discovered in various solid malignancies including breast, brain, prostate, and lung cancers . Transcriptional regulatory networks consist of functional interactions between regulatory genes and a much larger set of downstream target genes. Sox-2 was one of four transcription factors shown to reprogram mammalian somatic cells into induced pluripotent stem (iPS) cells. In the context of cancer, Sox-2 is over expressed in cervical, ovarian, lung, liver and breast carcinomas, and is a predictor of increased malignancy and poor prognosis. Although the importance of Sox-2 for ES cell maintenance and induction of iPS cells has been clearly demonstrated, it is unknown whether Sox-2 plays a role in regulating breast cancer stem cells .

Stem cells are concentrated in areas rich in blood vessels and stromal cells, in regions referred to as the 'stem cell niche'. This niche is thought to protect stem cells from apoptotic stimuli and to enable proper balance between self-renewal and differentiation. In fact, it was suggested that CSCs in the tumor microenvironment (TME) reside in a niche that is critical for their maintenance, similar to normal stem cells. This theory implies that disruption of the CSC niche, and thus stem cell maintenance, may provide an avenue of attack for eliminating CSCs.

TAMs constitute a major cell population in the breast TME. which secrete growth and other factors that permeate the breast stem cell niche to promote survival and self-renewal of stem cells. TAMs coexist in tumors and function as an accomplice to promote tumor progression and metastasis, especially once programmed and polarized into a proangiogenic/immune-suppressive(M2-like) phenotype by the TME. We previously reported that Fra-1 has a key role in the polarization of TAMs from the M1 to the M2 phenotype. In other words, expression of Fra-1 could be up regulated in TAMs that responded to stimulation by the TME, leading to an increased proportion of M2- like TAMs. Recently, it was reported that Fra-1 is indeed a miRNA target that may control tumor invasion and migration. However, it is currently unclear if miRNA plays a role in regulation of Fra-1 gene expression of TAMs in the TME.

2. KEYWORDS:

Cancer stem cells (CSCs); tumor-associated macrophages (TAMs); tumor microenvironment (TME); breast cancers epidermal growth factor receptors (EGFR); signal transducer and activator transcription 3 (Stat3); side population (SP); transcription factor Sox-2; EGFR/Stat3/Sox-2 signaling pathway; crosstalk between macrophages and breast cancer cells; micro-RNA (miRNA).

3. OVERALL PROJECT SUMMARY:

TAMs mediate cancer stem cells maintenance in vivo.

Interactions between CSCs and cells within their niche in the TME appear to be important for their maintenance and function. In order to assess the impact of TAMs on breast CSC maintenance and tumorgenicity *in vivo*, we injected mouse breast cancer stem cells 4T07-SP into syngeneic Balb/c mice that were either depleted or not of macrophages (Fig. 1A-B). Analysis of mice 25 d after tumor cell implantation revealed significantly reduced primary tumor and lung weights in animals, indicating reduced tumorigenicity and metastatic potential, respectively, in mice depleted of macrophages (Figs. 1C -D). Importantly, this finding correlated with a significant decrease in the percentage of 4T07- SP cells in primary tumors (Fig 1E). Taken together, these results indicate that TAMs mediate maintenance of SP cell populations *in vivo*, and that TAMs within the stem cell niche my play a critical role in CSC maintenance.

To further elucidate the mechanism of TAM-mediated regulation of breast CSCs, we performed a series of *ex vivo* experiments. We first analyzed the effects of RAW macrophage cell and tumor cell co-culture on the SPs of 4TO7 tumor cells. Co-culture of tumor cells with RAW cells resulted in a 2.8-fold increase in percentage of SP cells (Fig. 2A), which correlated with a marked increase in expression of

Sca-1 and AbcG2 on the surface of these cells (Fig. 2B). Macrophage co-culture resulted in increased mRNA and protein expression of Sox-2, Oct-4 and Nanog by SP cells (Figs. 2 C-D). Further analysis of the expression of Sox-2, Oct-4 and Nanog in primary tumors revealed that these key transcription factors were expressed by tumor cells proximal to TAMs (Figs. 2E-F). Together, these results suggest that TAMs actively communicate with tumor cells to enhance their expression of Sox-2 and other key regulators of CSC phenotypes. These findings further imply that TAMs regulate breast CSCs by mediating their expression of Sox-2 through paracrine signaling within the stem cell niche.

Sox-2 transcription factor regulates the tumorigenicity of breast cancer CSC.

Transcriptional regulation plays a key role in maintenance of cancer stem cell properties and the Sox-2 transcription factor was reported to be important for regulating ES cells. Therefore, we postulated that the Sox-2 signaling pathway might be involved in networks controlling breast CSC maintenance. We used siRNA to silence Sox-2 gene expression in 4TO7 cells (Fig. 3A). Intriguingly, we found that this also resulted in decreased Oct-4 and Nanog mRNA expression (Fig. 3B). Furthermore, expression of Sca-1 and AbcG2 stem cell markers was also markedly suppressed in the SP population of 4TO7 cells treated with Sox-2 siRNA (Fig. 3C). *In vivo*, Sox-2 knockdown in 4TO7 cells markedly suppressed tumor growth (Fig. 3D), reduced tumor weights (Figure 3E), and decreased lung metastasis, as indicated by decreased lung weights (Fig. 3F). *Ex-vivo* transwell migration assays showed that Sox-2 silencing also suppressed tumor cell motility (Fig. 3G). Additionally, Sox-2 silencing in SP cells not only increased tumor cell apoptosis, but also increased the sensitivity of these cells to mitoxantrone chemotherapy (Fig. 3H). Taken together, these data confirm that transcription factor Sox-2 is important for the maintenance of CSC phenotypes in murine breast cancer cells.

TAM activation of EGFRs on CSCs results in increased Sox-2 expression by tumor cells which is inhibited by EGF neutralizing antibody and EGFR inhibitor AG1478.

We next aimed to identify the signaling molecules responsible for TAM-mediated upregulation of Sox-2 expression by breast cancer cells. To determine whether soluble EGF released by TAMs mediates acquisition of CSC phenotypes by breast cancer cells, we examined expression of EGFR and phosphorylated EGFR (pEGFR) on 4T1 cells co-cultured with RAW macrophages. Interestingly, data showed over-expression of both EGFR and pEGFR by SP cells, compared with Non-SP cells, in pure 4T1 cultures (Fig. 4A). Moreover, this expression of EGFR and pEGFR by SP cells was further enhanced by co-culture with RAW cells, and correlated with an increase in percentage of SP cells (Fig. 4A). Similarly, culture of tumor cells with recombinant mouse EGF (mEGF) also induced an increase in the percentage of SP cells and a concordant increase in EGFR and pEGFR expression by these cells (Fig. 4B). Importantly, mEGF treatment resulted in increased expression of Sox-2 mRNA and protein by 4T1 tumor cells (Fig. 4C). This increase in Sox-2 expression also correlated with increased tumor cell migration upon mEGF stimulation (Fig. 4D). Moreover, analysis of protein expression of 4T1 cells that had been co-cultured, with either fresh isolated TAMs or RAW cells, demonstrated that expression of stat3, pstat3 or Sox-2 was down regulated after treatment with EGF neutralizing antibody (Fig. 4E). Furthermore, migration of 4T1 cells was suppressed by treatment with EGF neutralizing antibody (Fig. 4F). Together these data show that TAMs mediate effects which promote maintenance of SP cancer stem cell-like phenotypes supported by EGF release.

The requirement of EGFR activation for TAM/CSCs crosstalk and upregulation of Sox-2 expression by tumor cells was confirmed by blocking EGFR kinase activity with the inhibitor, AG1478. Treatment of 4T1 cells with AG1478 effectively blocked mEGF-induced upregulation of Sox-2 at both the mRNA and protein levels (Fig. 5A). Importantly, the increase in percentage of SP cells and expression of EGFR and pEGFR observed when 4T1 cells were cultured with RAW macrophage conditioned medium (MCM) was also completely blocked by AG1478 (Fig. 5B). Furthermore, analysis of mRNA and protein from these cells showed that AG1478 inhibited MCM-induced upregulation of both Sox-2 mRNA and protein expression (Fig. 5C). Inhibition of Sox-2 and EGFR expression by AG1478 was also confirmed by immunohistochemistry in 4T1 cells co-cultured with either RAW macrophages or RAW MCM (Figs. 5 D-

E). Together, these data show that TAM-mediated upregulation of Sox-2 expression in breast cancer cells is dependent on EGF/EGFR paracrine signaling.

EGFR-mediated upregulation of Sox-2 in breast cancer cells is Stat-3 dependent and can be blocked by Stat3 inhibitor.

EGF is a known ligand for the Signal Transducers and Activators of Transcription 3 (Stat3) signaling pathway . Upon binding of EGF to EGFR, Stat3 is phosphorylated which allows dimerization and translocation of the Stat3 protein into the nucleus where it activates transcription of downstream target genes. We hypothesized here that TAMs might utilize a similar paracrine signaling pathway via EGFR/Stat3 to regulate Sox-2 expression in breast cancer cells. Indeed, treatment of 4T1 cells with EGF induced phosphorylation of Stat3 and nuclear accumulation of Stat3 in a dose dependent manner (Fig. 6A). In contrast, treatment of tumor cells with the Stat3 inhibitor, CDDO-Im, blocked EGF-induced phosphorylation of Stat3 (Fig. 6B). Critically, EGF treatment significantly increased the percentage of SP cells in 4T1 tumor cell cultures, an increase which was also effectively blocked by CDDO-Im (Fig. 6C). Furthermore, RT-PCR and western blot analysis indicated that treatment of 4T1 cells with CDDO-Im also blocked EGF-induced upregulation of Sox-2 mRNA and protein expression (Figures 6D and E). Additionally, partial inhibitions of Oct-4 and Nanog mRNA and protein expression were also observed following CDDO-Im treatment (Figs. 6D-E).

Since we found that CSC phenotypes can be induced by MCM and blocked by inhibiting EGFR tyrosine kinase activity via AG1478, we next determined whether these phenotypes could also be blocked through Stat3 inhibition by CDDO-Im. To this end, we treated 4T1 cells with EGF or IL-6 in combination with CDDO-Im. Western blot analysis revealed that only EGF treatment resulted in an increase in nuclear Stat3 and Sox-2 protein expression, and was inhibited by CDDO-Im (Fig. 6F). Interestingly, EGF treatment also induced a decrease in E-cadherin and an increase in N-cadherin expression in 4T1 cells (Fig. 6F). This observation is significant since changes in cadherin expression have been linked to de-differentiation of breast cancer cells and association with acquisition of CSC phenotypes. Similar to EGF, incubation of breast cancer cells with MCM also resulted in nuclear accumulation of Sox-2 protein (Fig. 6G). Additionally, nuclear accumulation of β-catenin, and a concomitant decrease in cytoplasmic β-catenin, protein expression was also observed in response to culture with MCM (Fig. 6G). Importantly, both upregulation of Sox-2 expression and nuclear translocation of β-catenin in response to MCM could be inhibited by treatment with CDDO-Im (Fig. 6G).

Because these changes in E- and N-cadherin expression and β-catenin localization are associated with increased cell motility, we investigated the effects of MCM on tumor cell motility. We found that transwell migration of both 4T1 and 4TO7 breast cancer cells was significantly increased after culture with MCM (Fig. 6H). Importantly, this MCM-induced migration of tumor cells was significantly inhibited by CDDO-Im (Fig. 6I).

Tumor microenvironment changes miRNAs expression patterns and downregulation of miR-19a-3p enhance M2-like phenotpye of macrophage by upregulation of Fra-1/Stat3 pathway activity.

A search for potential microRNAs that target 3'URT of Fra-1, as a proto-oncogene which play important role in modeling tumor microenvironment, was based on the miRBase and TargetScan software prediction. Expression of miR-19a-3p was down regulated significantly in RAW cells after culturing with 4T1 conditional medium of three candidates (miR-19a-3p, miR-29a and miR-503) (Figs. 7A -B). To investigate the miRNA expression patterns in TAMs *in vivo,* they were induced by the TME. The miRNA profile detection was performed. The data indicate that miR-19a-3p expression was significantly decreased in (Fig. 7C). Taken together, these results suggest that the 3'UTR of Fra-1 gene is a potential target for miR-19a-3p which can regulate *Fra-1* gene expression in TAMs within the breast tumor TME. Furthermore, we observed the opposite of the relationship between Fra-1 and miR-19a-3p expression of TAMs. This was implied that the 3'UTR of Fra-1 could be a potential target of miR-19a-3p. To confirm this, the miR-19a-3p mimic was synthesized and transfected into RAW cells. In this case, Fra-1 expression was significantly suppressed by the miR-19a-3p mimic within 24 hours (Figs. 7D-E). To

further explore whether miR-19a-3p could regulate the downstream, STAT3 pathway, of Fra-1 via inhibition of Fra-1 expression, miR-19a-3p mimic was synthesized, and then transfected into RAW cells respectively. In this case, the expressions of pSTAT3, STAT3 as well as their target gene VEGF-A were either suppressed (Fig. 7F). Together, these findings indicate that miR-19a-3p regulates expression of Fra-1 and activity of its downstream pathway. The miR-19a-3p also down-regulates some other signal pathways that promote M2 polarization of macrophages.

Down-regulated miR-19a-3p promotes M2 phenotype of TAMs via releasing the Fra-1/STAT3 pathway and inhibiting metastasis of 4T1 breast cancer cell by suppressing M2 macrophage function

Polarization of M1 to M2 phenotype is an important transformation of macrophages in the TME. In order to prove that the regulation of miR-19a-3p may support maintenance of the M2 phenotype of TAMs in the breast tumor TME, an analysis of M1 or M2 marker expression was performed among mouse primary macrophages or RAW cells transfected with the miR-19a-3p mimic. Results showed that expression of M2 markers- Arg1 and CD206 were significantly decreased. Thus, reverse M1 marker-Mcp1 and Nos2 expressions were increased in macrophages that were transfected with the miR-19a-3p mimic (Fig.8A). We determined whether inhibition of Fra-1 by miR-19a-3p in RAW cells could indeed impair invasion and migration capacity of breast tumor cells in the TME. To this end, three mouse breast tumor cell lines, 4T1, 4T07 and EMT6 were used as following: transwell assays indicated that invasion capacity of these three tumor cell lines were significantly suppressed when co-cultured with RAW cells, after transfectionwith miR-19a-3p (Fig. 8B).

Furthermore, mice xenografted with 4T1 cells were injected twice a week intratumorally with miR-19a-3p agomir, a chemically modified miRNA mimic. Tumor mass and lungs from these mice were harvested after three weeks (Fig. 9A) and the CD206 positive cells in tumor tissue were significantly decreased after injection with miR-19a-3p agomir (Figs. 9B). We determined whether miR-19a-3p inhibited the growth and metastasis of 4T1 breast tumor by suppressing macrophages with M2 phenotype. To this end, measurement of the tumor volume and lung metastasis in balb/c mice, resulted in a significantly attenuated metastasis capacity of tumor cells, but not of original tumor growth (Fig. 9C-D) after injecting miR-19a-3p agomir into our 4T1 tumor model. Together, our data indicate that miR-19a-3p is down regulated in macrophages with M2 phenotype. The low expression of miR-19a-3p apparently plays an important role in up regulation of Fra-1 expression and induction in M2 macrophage polarization which may contribute considerably in facilitating metastasis and progression of breast cancer.

4. KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstrated a key role for tumor-associated macrophages (TAMs) in promoting CSC phenotypes in murine breast cancer cells.
2. Identified a novel mechanism of regulation achieved by paracrine EGF signaling between TAMs and breast cancer cells. Established that signaling between TAMs and tumor cells involves activation of the EGFR/Stat3 signaling pathway and the downstream upregulation of transcription factor Sox-2.
3. Importantly, crosstalk between TAMs and tumor cells was found to require EGF-R and Stat3 activation that could be blocked by small molecule inhibitors of either EGF-R or Stat3. This establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for cancer stem cell therapy.
4. Demonstrated that inhibiting miR-19a-3p expression in the tumor microenvironment activates the Fra-1/IL-6/STAT3 signaling pathway of TAMs. Afterwards, TAMs are polarized to the M2 phenotype which effectively promotes invasive, metastatic and angiogenetic capacities of tumor cells.

5. CONCLUSION:

To our knowledge, we are the first to describe a unique interaction between TAMs and breast cancer

cells via EGF/EGFR/Stat3 signaling which is critical for expression of transcription factor Sox-2 and the maintenance of breast cancer stem cells. Importantly, we show that this cross-talk was effectively blocked by inhibition of either EGFR tyrosin kinase inhibitor AG1478 or Stat3 inhibitor CDDO-Im. We identified a novel role for macrophages in the regulation of breast cancer stem cells which establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for breast cancer stem cell therapy.

We identified the microRNA expression pattern in TAMs induced by stimuli from the TME in a mouse breast tumor model. Furthermore, our study demonstrated an important normal mechanism by which miRNAs could induce the phenotype switch between TAMs and normal macrophages by regulating the expression of the Fra-1 gene and the Fra-1/STAT3 signaling pathway. These findings aided us in exploring the potential of therapy targets on miRNA capable of regulating the switching of the TAM phenotype, resulting in remodeling of the TME.

6. PUBLICATIONS, ABSTACTS AND PRESENTATIONS:

Poster presentation:
1. AACR Annual Meeting at Chicago, IL (2012), Abstract #3366 in the TB02-05 Cancer stem Cell session on 4/2/12. The presentation was entitled “An inhibitor of the NF-kB pathway targets cancer stem cells and prevents tumor recurrence”.
2. AACR Annual Meeting at Washington DC (2013), Abstract # 2828. The presentation was entitled “miR-19a-3p inhibits breast carcinoma metastasis via reversing M2 phenotype of TAMs".

Publications:

1. Yang J, Liao D, Chen C, Liu Y, Chuang TH, Xiang R, Markowitz D, Reisfeld RA, Luo Y. Tumor associated macrophages regulate murine breast cancer stem cells through a novel paracrine EGFR/Stat3/Sox-2 signaling pathway. Stem Cells. 2012 Nov 21. doi: 10.1002/stem.1281. [Epub ahead of print]
2. Yang J, Zhang A, Chen C, Liu Y, Si Q, Chuang TH, li N, Gomez-Cabrero A, Reisfeld RA, Xiang R, and Luo Y* MicroRNA-19a-3p inhibits breast cancer progression and metastasis by inducing Macrophage polarization through down-regulated expression of Fra-1 proto-oncogene. Oncogene. 2013, online

7. INVENTIONS, PATENTS AND LICENSES:

None.

8. REPORTABLE OUTCOMES:

1. Yang J, Liao D, Chen C, Liu Y, Chuang TH, Xiang R, Markowitz D, Reisfeld RA, Luo Y. Tumor associated macrophages regulate murine breast cancer stem cells through a novel paracrine EGFR/Stat3/Sox-2 signaling pathway. Stem Cells. 2012 Nov 21. doi: 10.1002/stem.1281. [Epub ahead of print]
2. Yang J, Zhang A, Chen C, Liu Y, Si Q, Chuang TH, li N, Gomez-Cabrero A, Reisfeld RA, Xiang R, and Luo Y* MicroRNA-19a-3p inhibits breast cancer progression and metastasis by inducing Macrophage polarization through down-regulated expression of Fra-1 proto-oncogene. Oncogene. 2013, online

9. OTHER ACIEVEMENTS: N/A

10. REFERENCES: N/A

11. APPENDICES: N/A

Figure legends

Figure 1. TAMs mediate SP cell maintenance *in vivo*. (A) 4TO7-SP cells were isolated by HOECHST 33342 dye staining and flow cytometry cell sorting. These SP cells ($1x10^3$) were then injected into Balb/c mice that had previously been depleted of macrophages by treatment with cholondrite NPs (M φ KO). Control animals were treated with saline and thus not depleted of macrophages (WT). (n=5 mice/group) **(B)** Macrophage populations ($CD45^+/F4/80^+$) in blood and primary tumors of WT or M φ KO mice were measured by flow cytometry. Data represent means±S.E.M. Mice were sacrificed 25 d after SP cell challenge and tumor **(C)** and lung **(D)** weights were measured. Data represent means±S.E.M. **(E)** The percentages of SP cells in primary tumors from WT and M φ KO mice were measured by HOECHST 33342 dye staining and flow cytometry. Data represent means±S.E.M. *$p<0.05$, **$p<0.005$.

Figure 2. TAMs enrich SP cells and enhance expression of Sox-2, Oct-4 and Nanog in breast cancer cells. (A) The SP of 4T07 breast cancer cells was determined by Hoechst staining and Flow cytometry after 96 h of co-culture with either TAM derived from 4T07 tumor tissue or RAW macrophages. (B) Expression of Sca-1 and ABCG2 was also detected in this same population. (C) Expression of Sox-2, Oct 4 and Nanog was determined by RT-PCR, and (D) by Western blot. (E, F) Expression of Sox-2, Oct 4 and Nanog in 4T07 tumor tissue was confirmed by Immunofluorscence histology staining. Scale bars, 100μm; 150 μm on lower panel of E.

Figure 3. Transcription factor Sox-2 regulates maintenance of cancer stem cell-like properties of SP cells. Sox-2 gene expression in 4TO7 cells was silenced using siRNA. **(A)** Down regulation of Sox-2 was confirmed by RT-PCR. **(B)** Gene expression of Sox-2, Oct-4 and Nanog by SP cells was determined by RT-PCR after Sox-2 silencing. **(C)** Expression of surface makers Sca-1 and ABCG2 was determined by flow cytometry in 4TO7 SP cells after Sox-2 silencing. **(D)** Balb/c mice were challenged with 4TO7-SP wildtype (WT) or 4TO7-SP cells subjected to Sox-2 silencing (Sox-2 siRNA) and tumor volumes were measured every 3-4 d. (n=5mice/group). 25 d after tumor cell challenge, tumor **(E)** and lung **(F)** weights were measured. Data represent means±S.E.M. **(G)** Migraion assays were performed on 4TO7 WT or Sox-2 siRNA-treated SP cells using Boyden transwell chambers. (n=3 wells/group). **(H)** Effects of Sox-2 silencing on apoptosis of Non-SP and SP cells were determined by Annexin V staining and flow cytometry. Additionally, the sensitivity of SP and Non-SP cells to mitoxantrone chemotherapy was also assessed after Sox-2 knockdown.

Figure 4. TAMs and EGF induce overexpression of EGFR and pEGFR on SP cells that correlates with increased Sox-2 expression and cell motility, which are inhibited by EGF neutralizing antibody . The expression of EGFR or phosphorylated EGFR (pEGFR) was detected by HOECHST 33342 dye and antibody staining, followed by flow cytometry analysis in 4T1 SP and Non-SP cells after co-culture with RAW macrophages (A) or recombinant mouse EGF (mEGF) (B). (C) Expression of Sox-2 at the mRNA (left panels) and protein (right panels) levels in 4T1 cells was assessed after treatment with mEGF. (D) EGF-induced migration of 4T1 cells was determined using Boyden transwell chambers Data represent means±S.E.M. The expression of Sca-1 and ABCG2 in SP population of 4T1 cells was determined after co-cultured with RWA cells and treated by EGF neutralizing antibody. (E) Either TAMs from 4T1 tumor tissue or RAW cells- induced overexpression of Stat 3, pStat3 and Sox-2 was inhibited by EGF neutralizing antibody in 4T1 cells. (F) Migration of 4T1 tumor cell after co-cultured with RAW cell was also inhibited by EGF-Ab. Data represent means±S.E.M. (3 wells/group).

Figure 5. Macrophage mediated effects on breast cancer cells are inhibited by EGFR inhibitor AG1478. (A) EGF-induced overexpression of Sox-2 was inhibited in 4T1 cells by AG1478, at both the mRNA (left panels) and protein (right panels) levels. (B) Culture of 4T1 cells with RAW macrophage conditioned medium (MCM) increases the percentage of SP cells and correlates with increased EGFR and

pEGFR expression as determined by HOECHST 33342, antibody staining and flow cytometry. **(C)** MCM-induced increases in Sox-2 expression are inhibited by AG1478 at both the mRNA (left panels) and protein (right panels) levels. Inhibition of Sox-2 and EGFR expression after AG1478 treatment was confirmed by immunohistochemistry in 4T1 cells co-cultured with either RAW macrophages **(D)** or MCM **(E)**. Scale bar, 100μm.

Figure 6. EGF-induced upregulation of SP cells is Stat3 dependent. (A) Western blot analysis of cell lysates from 4T1 cells treated with EGF at varying concentrations for 15 m. **(B)** Western blot analysis of cell lysates from 4TO7 cells treated with EGF (20ng/mL) plus CDDO-Im at varying concentrations for 10 m. **(C)** 4T1 cells treated with EGF, with or without CDDO-Im, were analyzed by HOECHST 33342 dye staining and flow cytometry to quantify percentage of side population (SP) cells. Data represent means±S.E.M. *$p<0.05$. **(D-E)** Treatment of 4T1 cells with CDDO-Im (100nM) suppresses EGF-induced upregulation of Sox-2, Oct-4 and Nanog mRNA **(D)** and protein **(E)** expression, evaluated by RT-PCR and Western blotting, respectively. **(F)** Western blotting of lyastes from 4T1 cells treated with either IL-6 or EGF in combination with CDDO-Im. **(G)** Western blotting of lysates from 4T1 cells cultured in macrophage conditioned medium (MCM), with or without CDDO-Im. **(H-I)** Migration of 4T1 and 4TO7 breast cancer cells in response to incubation with MCM, either with or without CDDO-Im, was determined using Boyden transwell chambers and quantified with ImageJ software (3 wells/group). *$p<0.05$, **$p<0.005$.

Figure 7. Tumor microenvironment changes miRNAs expression patterns and low level expression of miR-19a-3p up-regulates expression of the Fra-1 and Fra-1/Stat 3 signaling pathways in macrophages. (A) Prediction of Fra-1 3'UTR binding by miRNAs based on targetscan online software. (B) miR-19a-3p, miR-29a and miR-503 expression by RAW cells, co-cultured with 4T1 conditional medium, as detected by real-time PCR, (n=3 *** $p<0.001$}. (C) Total RNA was collected from TAMs and normal macrophages derived from mouse 4T1 breast cancer tissue and spleen respectively, and miRNA expression was detected by real-time PCR. (D) Expression of Fra-1 in RAW cells that were treated with miR-19a-3p mimic for 24 or 48 hours (E-F) MiR-19a-3p mimic and inhibitor were synthesized and transfected into RAW cells respectively, and expressions of pSTAT3, STAT3 and VEGF-A detected by western blots.

Figure 8. miR-19a-3p down-regulates the M2 phenotype of macrophages via suppression of the Fra-1/STAT3 pathway and invasion and capacities of RAW cells are inhibited by miR-19a-3p via. (A) Arg1, CD206, Mcp1 and Nos2 expression were detected in RAW cells transfected with miR-19a-3p mimic and compared with a negative control. (B) Invasion of 4T1, 4T07 and EMT6 mouse breast tumor cells was detected by the transwell assay.

Figure 9. miR-19a-3p inhibits breast cancer cell metastasis by regulating the M2 phenotype of TAMs in a Balb/c mouse breast tumor model. (A) 4T1 cells labeled with luciferase were incubated in Balb/c mice. These mice, xenografted with 4T1 cells, were injected intratumorally twice weekly with miR-19a-3p agomir or a negative control. (B) Tumor masses and lungs from these animals were harvested after three weeks and CD206 positive cells were detected after injection with miR-19a-3p agomir by immunohistochemistry. The lung metastases of balb/c mice (n=3, *** $p<0.001$). (C) and tumor volume (D) were measured after injecting miR-19a-3p agomir into the 4T1 tumor model.

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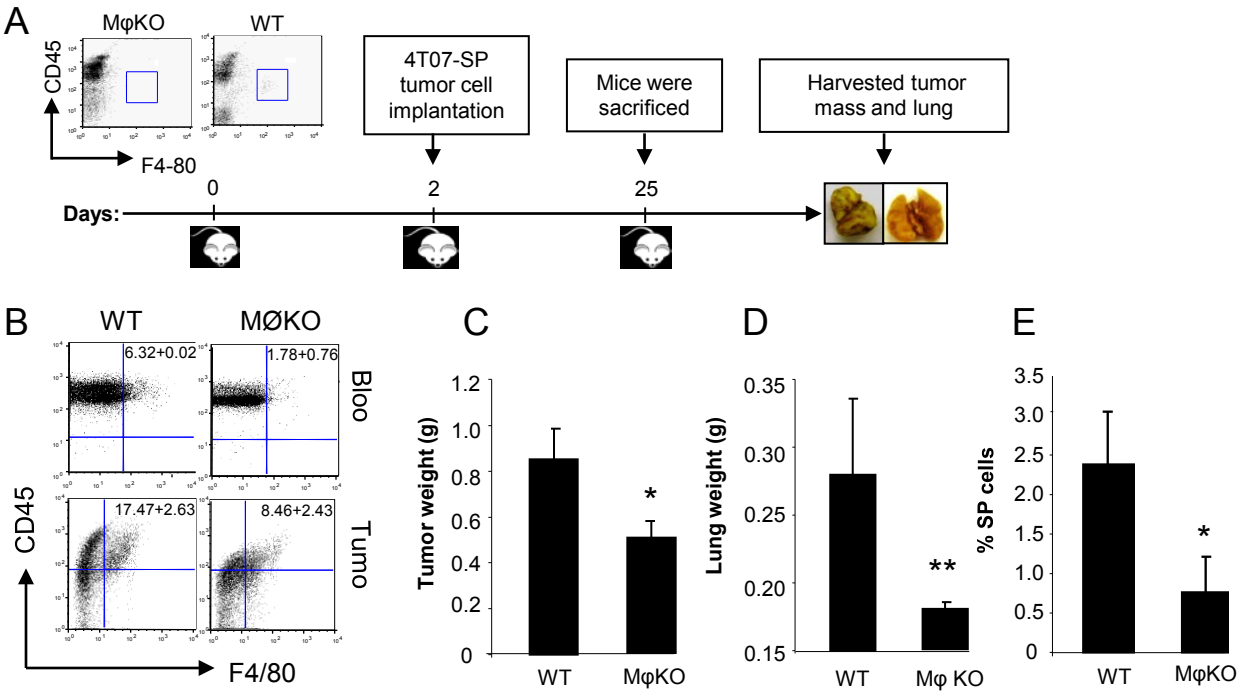

Figure 1. TAMs mediate SP cell maintenance *in vivo*.

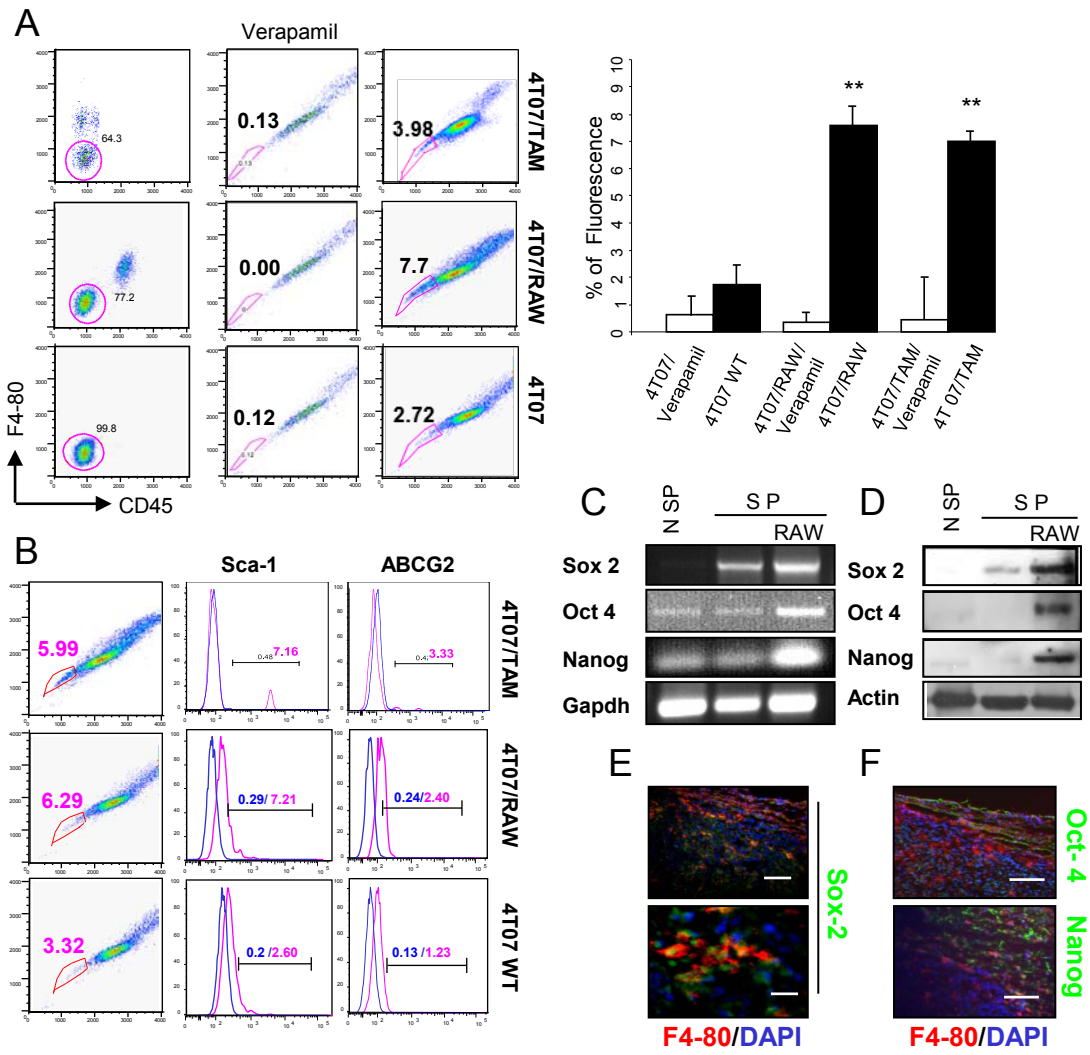

Figure 2. TAMs enrich SP cells and enhance expression of Sox-2, Oct-4 and Nanog in breast cancer cells.

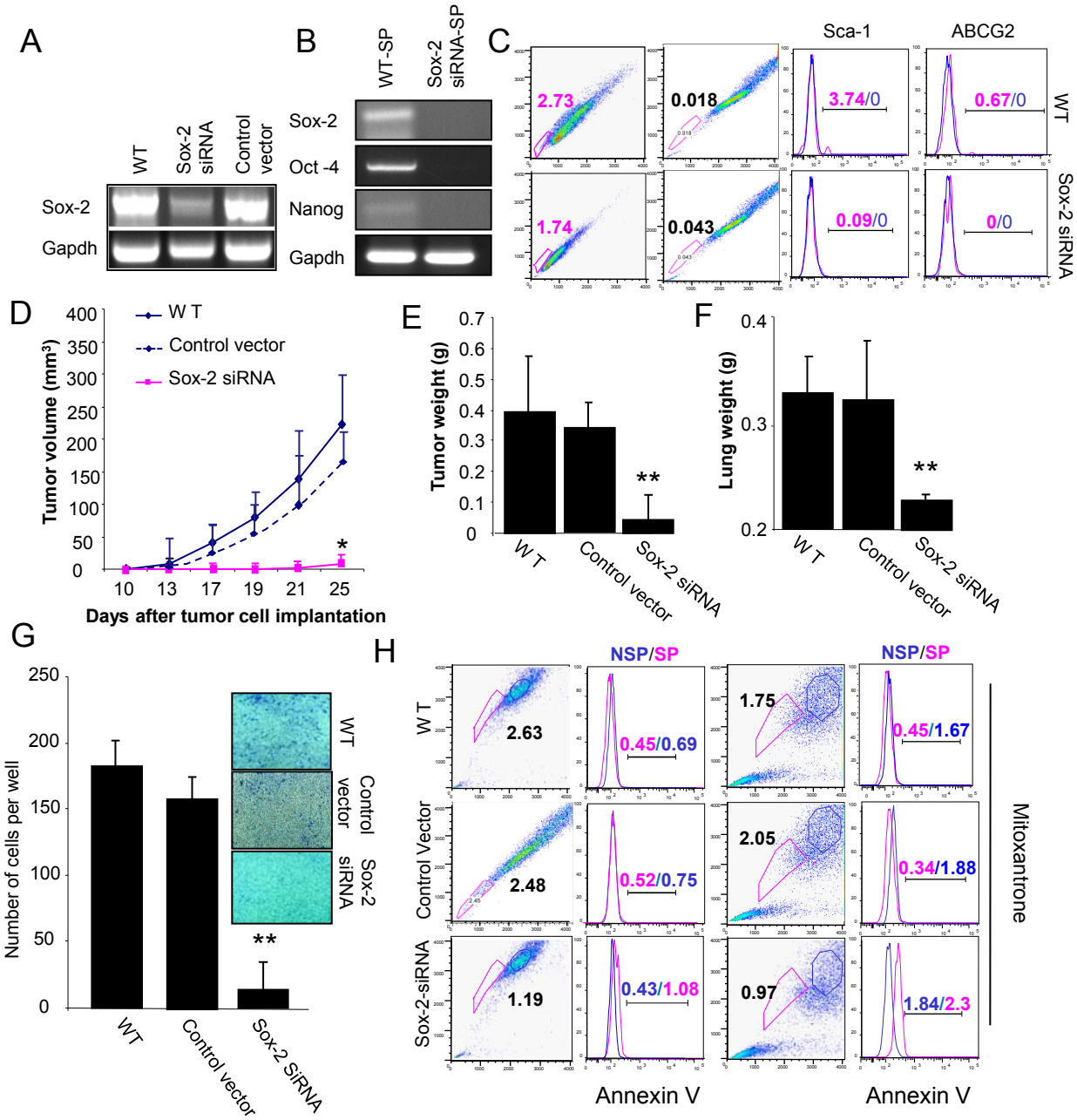

Figure 3. Transcription factor Sox-2 regulates the maintenance of cancer stem cell-like properties of SP cells.

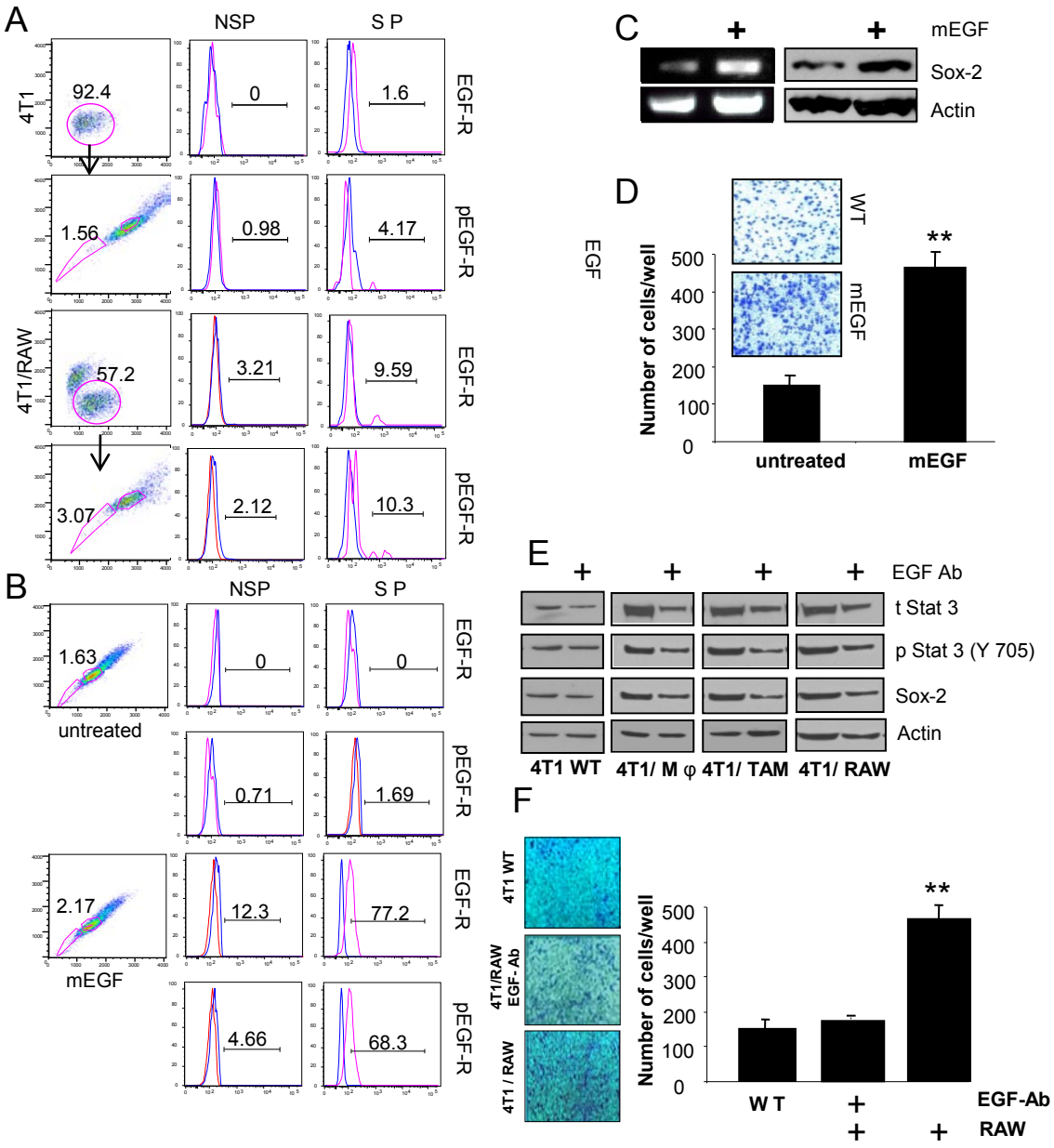

Figure 4. TAMs and EGF induce overexpression of EGFR and pEGFR on SP cells that correlates with increased Sox-2 expression and cell motility, which are inhibited by EGF neutralizing antibody .

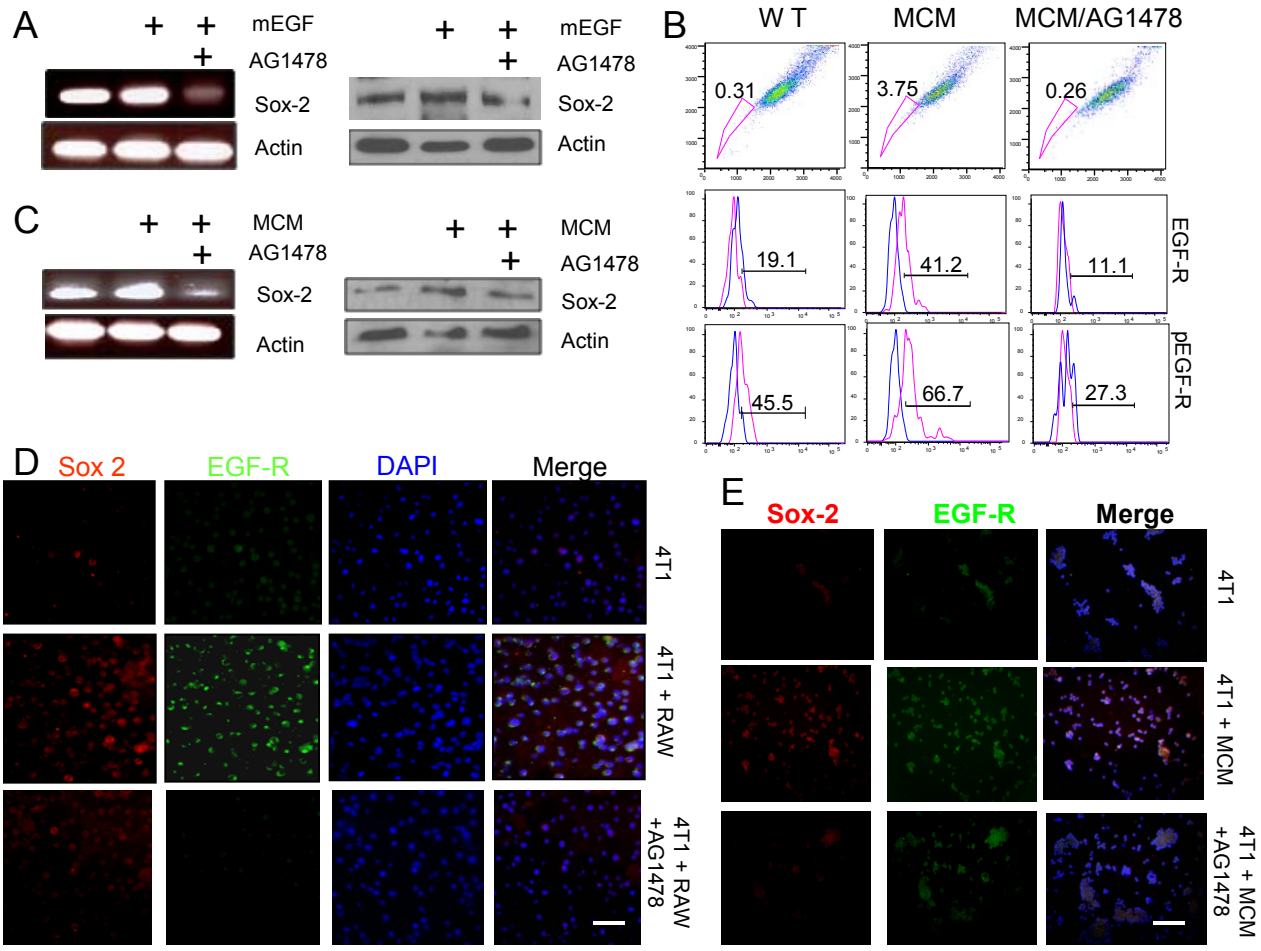

Figure 5. Macrophage mediated effects on breast cancer cells are inhibited by EGFR inhibitor AG1478.

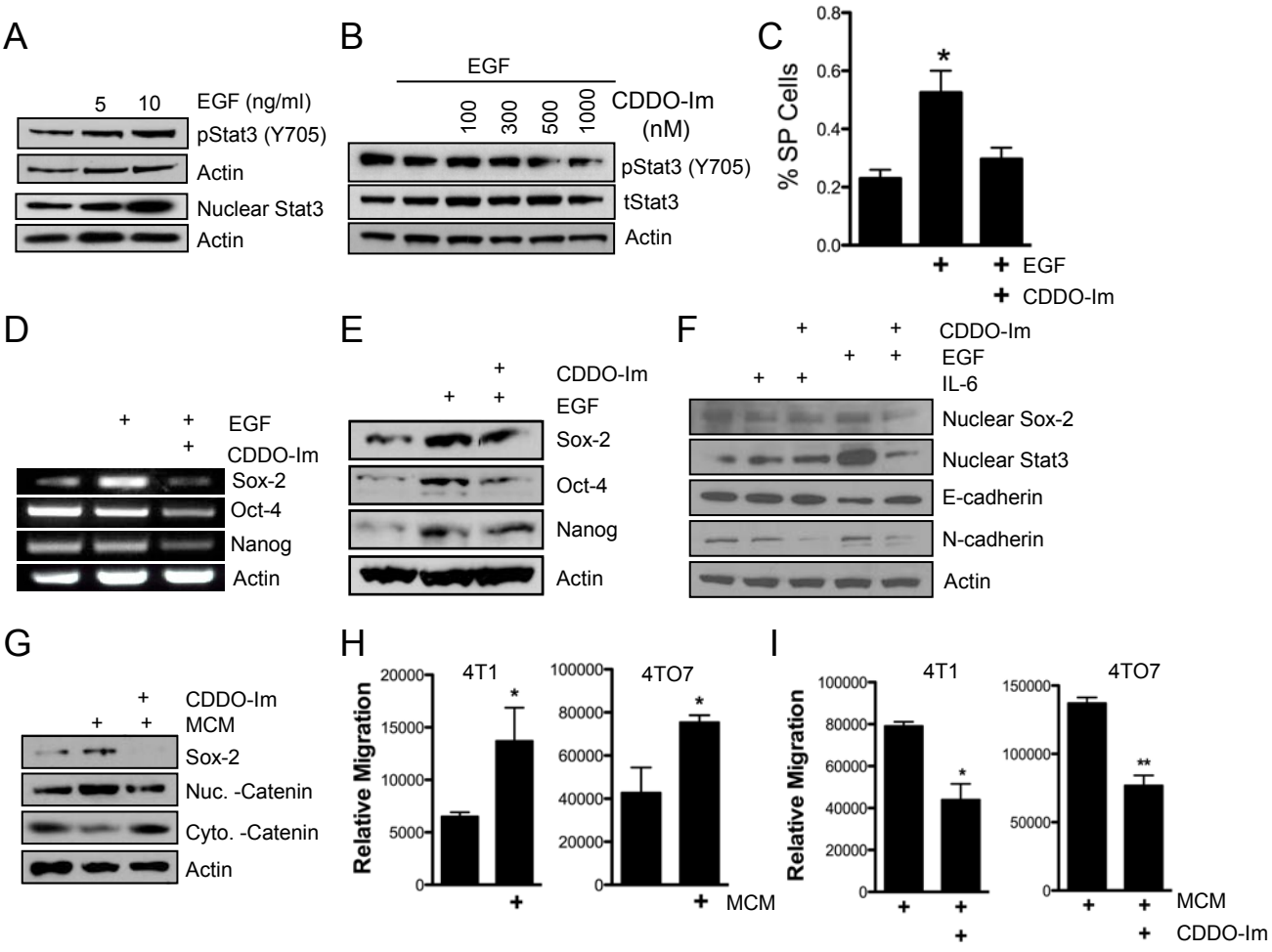

Figure 6. EGF-induced upregulation of SP cells is Stat3 dependent

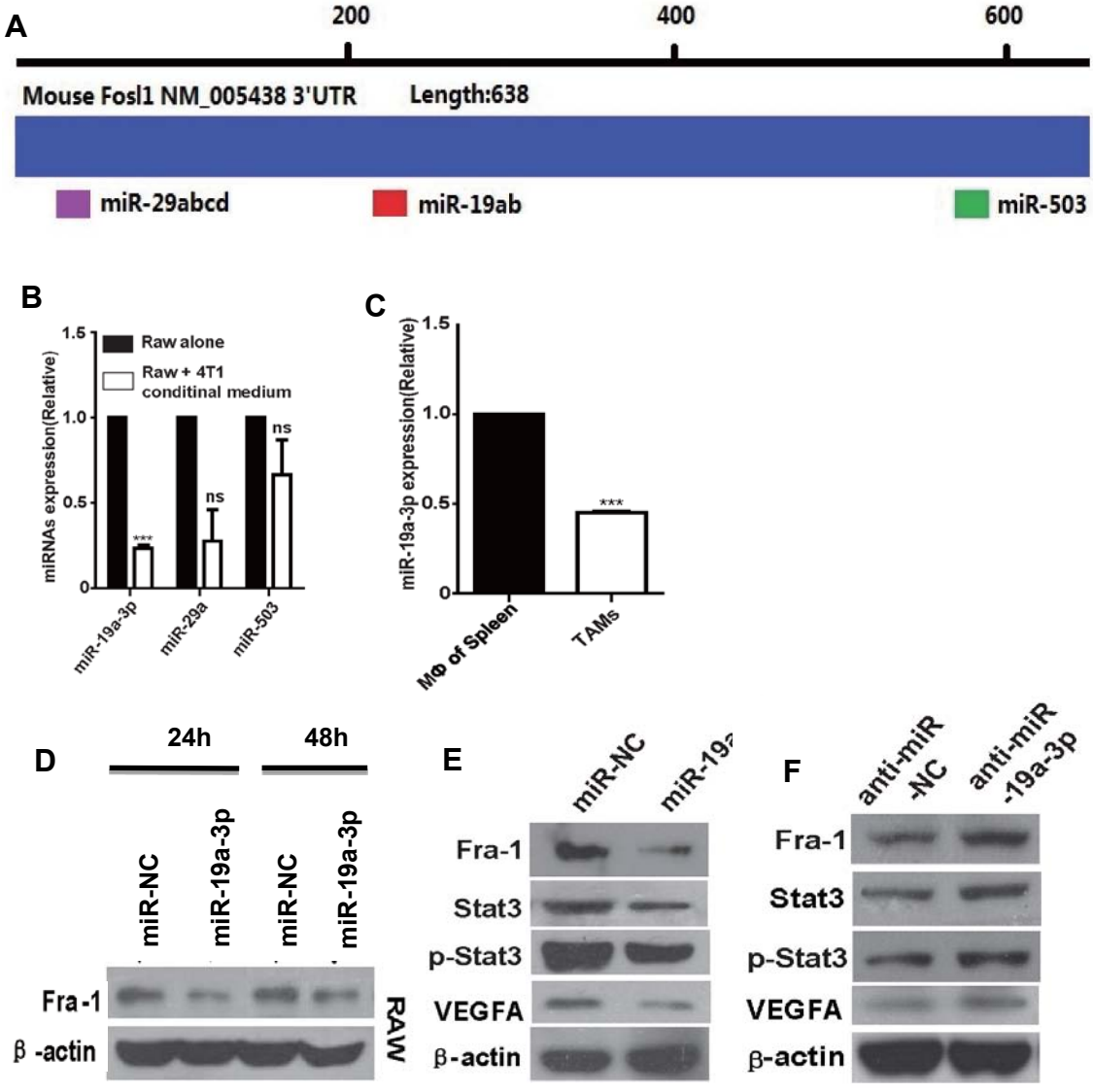

Figure 7. Tumor microenvironment changes miRNAs expression patterns and low level expression of miR-19a-3p up-regulates expression of the Fra-1 and Fra-1/Stat 3 signaling pathways in macrophages.

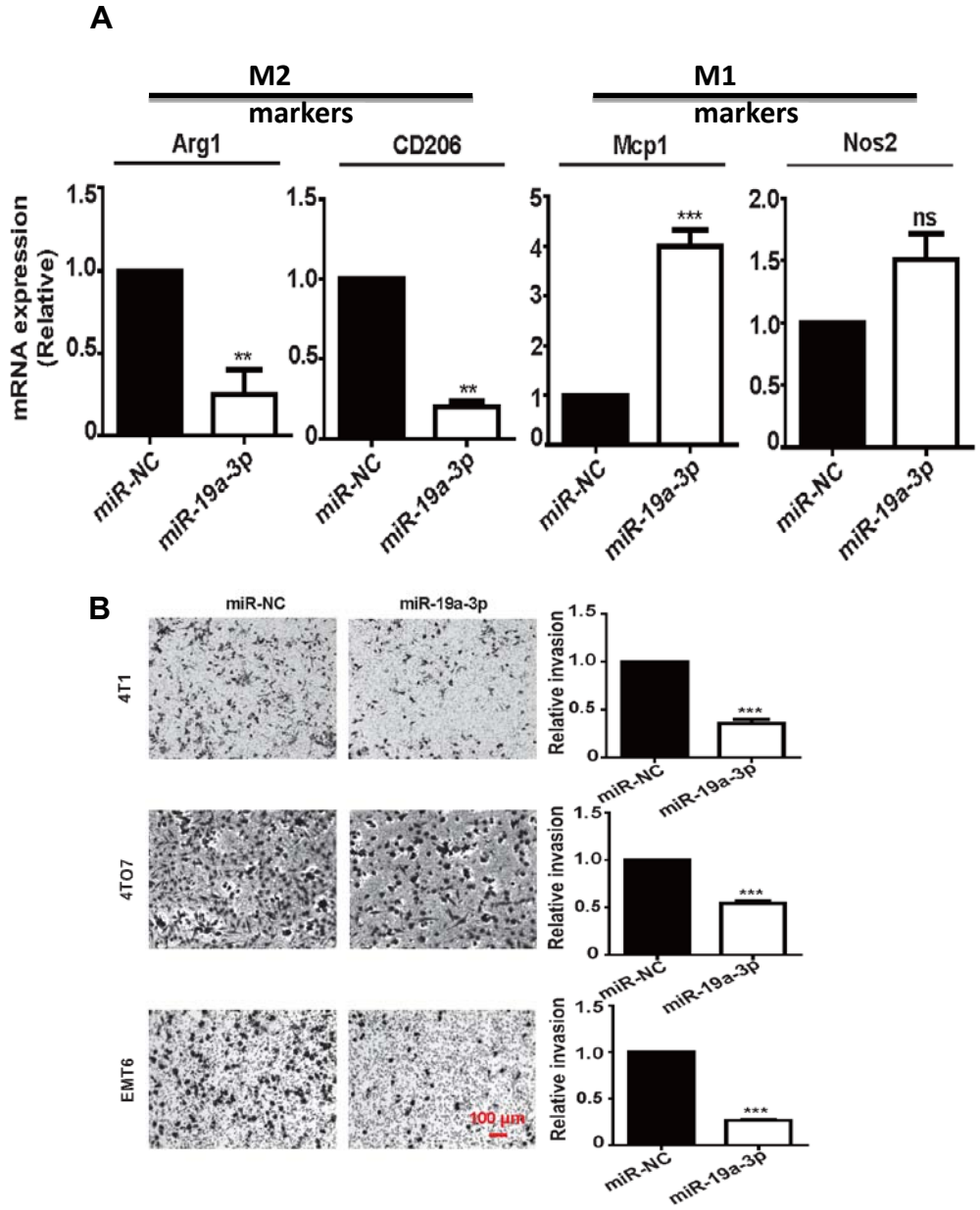

Figure 8. miR-19a-3p down-regulates the M2 phenotype of macrophages via suppression of the Fra-1/STAT3 pathway and invasion and capacities of RAW cells are inhibited by miR-19a-3p via.

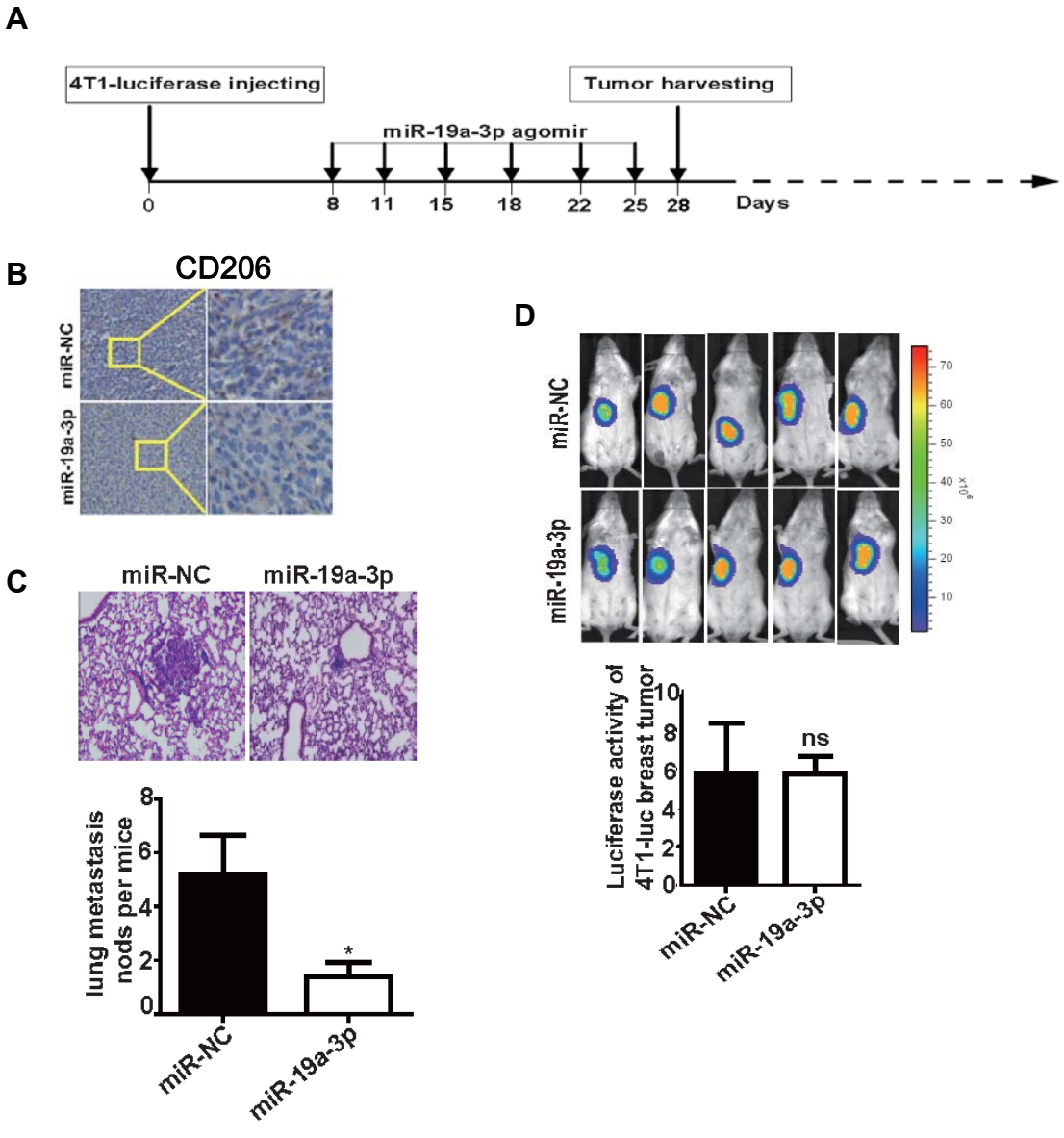

Figure 9. MiR-19a-3p inhibits breast cancer cell metastasis by regulating the M2 phenotype of TAMs in a Balb/c mouse breast tumor model.

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Presentation Abstract

Abstract Number:	3366
Presentation Title:	An inhibitor of the NF-kappaB pathway targets cancer stem cells and prevents tumor recurrence
Presentation Time:	Tuesday, Apr 03, 2012, 8:00 AM -12:00 PM
Location:	McCormick Place West (Hall F), Poster Section 14
Poster Section:	14
Poster Board Number:	11
Author Block:	Azucena Gomez-Cabrero, Debbie Liao, Giang Nguyen, Ralph Reisfeld. The Scripps Research Institute, La Jolla, CA
Abstract Body:	Multidrug resistance and tumor recurrence are two of the major challenges in cancer treatment. Cancer stem cells (CSCs), found in most solid tumor types, have been proposed as the root cause of cancer growth and recurrence. These CSCs share similarities with normal Stem Cells, such as "stemness" gene profile expression, overexpression of Sox2, Nanog and Oct4, as well as ability to grow in spheres and to repopulate tumors. Additionally, CSCs also have a high resistance to chemotherapeutic agents, probably due to their dormancy and overexpression of ABC transporters. Since CSCs markers are highly variable between cell lines and tumor samples, we used a functional assay to select a drug candidate targeting CSCs, based on their capability to efflux dyes such as Hoechst 33324 by a side population (SP). This drug decreased SP and spheres formation in vitro, and reduced protein expression of CSC genes such as Sox2, Nanog, Survivin and Oct4. Another serious limitation of cancer chemotherapy is its toxic side effect due to poor targeting to the tumor and its microenvironment (TME). To address this issue, we developed targeted nanoparticles (tNP) to deliver chemotherapeutic drugs specifically to tumor cells and their TME, resulting in substantially reduced systemic toxicity, as well as eradication of tumors and prevention of their recurrence in a preclinical setting.

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April 6-10, 2013, Walter E. Washington Convention Center, Washington, DC

Presentation Abstract

Abstract Number:	2828
Presentation Title:	miR-19a-3p inhibits breast carcinoma metastasis via reversing M2 phenotype of TAMs
Presentation Time:	Tuesday, Apr 09, 2013, 8:00 AM -12:00 PM
Location:	Hall A-C, Poster Section 20
Poster Board Number:	30
Author Block:	Jian Yang[1], Na Li[1], Zhuhong Zhang[1], Qin Si[2], Chong Chen[2], Yan Liu[2], Ralph A. Reisfeld[3], Peiqing Sun[3], Dwayne Stupack[4], Rong Xiang[1], Yunping Luo[2]. [1]Department of Immunology, School of Medicine, Nankai University, Tianjin, China; [2]Department of Immunology, Beijing Union Medical School, Beijing, China; [3]The Scripps Research Institute, Tianjin, CA; [4]Department of Pathology, School of Medicine, University of California San Diego, Tianjin, CA
Abstract Body:	Tumor-associated macrophages (TAMs), most of which exhibit M2 phenotype, function as immunosuppressive cells in tumor microenvironment (TME). Polarization of TAMs from a pro-immune (M1 like) phenotype to an immune-suppressive (M2-like) phenotype is one of the hallmarks of malignancy, but their molecular basis is still remains unknown. It has been reported that microRNAs are involved in monocyte-macrophage differentiation. In this study, we found that miR-19a-3p, broadly conserved in vertebrate, could reverse the M2 phenotype of RAW macrophage cells. When mouse breast tumor cells such as 4T1, 4TO7 and EMT6 were co-cultured with RAW macrophage cells which over express miR-19a-3p, the invasion capacity was suppressed. Meanwhile, when the conditional medium of RAW cells which were transfected with miR-19a-3p mimic was added into culturing medium of tumor cells, the migration capacity of 4T1 and EMT6 breast cancer cells was inhibited. Moreover, when miR-19a-3p was injected intratumor, consistent with the in vitro experiments, we found that the M2 phenotype of TAMs was suppressed significantly. Although 4T1 xengraft breast tumor growth was not affected by miR-19a-3p, lung metastasis of tumor cells was significantly suppressed. Taken together, our findings indicate that miR-19a-3p is down-regulated in M2 phenotype RAW macrophage and TAMs in TME. The low expression of miR-19a-3p plays an important role in inducing M2 macrophage polarization and promoting migration and invasion capacity and metastasis.

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Tumor-Associated Macrophages Regulate Murine Breast Cancer Stem Cells Through a Novel Paracrine EGFR/Stat3/Sox-2 Signaling Pathway

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Key Words. Breast cancer • Cancer stem cells • Side population cells • Stem cell-microenvironment interactions • Transcription factors • STAT

ABSTRACT

The cancer stem cell (CSC) hypothesis has gained significant recognition as a descriptor of tumorigenesis. Additionally, tumor-associated macrophages (TAMs) are known to promote growth and metastasis of breast cancer. However, it is not known whether TAMs mediate tumorigenesis through regulation of breast CSCs. Here, we report that TAMs promote CSC-like phenotypes in murine breast cancer cells by upregulating their expression of Sox-2. These CSC-like phenotypes were characterized by increased Sox-2, Oct-4, Nanog, AbcG2, and Sca-1 gene expression, in addition to increased drug-efflux capacity, resistance to chemotherapy, and increased tumorigenicity in vivo. Downregulation of Sox-2 in tumor cells by siRNA blocked the ability of TAMs to induce these CSC-like phenotypes and inhibited tumor growth in vivo. Furthermore, we identified a novel epidermal growth factor receptor (EGFR)/signal transducers and activators of transcription 3 (Stat3)/Sox-2 paracrine signaling pathway between macrophages and mouse breast cancer cells that is required for macrophage-induced upregulation of Sox-2 and CSC phenotypes in tumor cells. We showed that this crosstalk was effectively blocked by the small molecule inhibitors AG1478 or CDDO-Im against EGFR and Stat3, respectively. Therefore, our report identifies a novel role for TAMs in breast CSC regulation and establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for CSC therapy. STEM CELLS *2013;31:248–258*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Transcriptional regulatory networks consist of functional interactions between regulatory genes and a much larger set of downstream target genes. The network of Sox-2/Oct-4 target genes includes Sox-2, Oct-4, Nanog, FGF-4, UTF1, Fbx15, and Lefty1 [1–3]. Each of these genes is expressed in embryonic stem cells (ESCs) and was shown to be substantially downregulated upon differentiation of both ESCs and embryonic carcinoma cells due to decrease of Sox-2 and Oct-4 expression [1, 2]. Additionally, Sox-2 was one of four transcription factors shown to reprogram mammalian somatic cells into induced pluripotent stem (iPS) cells [4]. In the context of cancer, Sox-2 overexpression has been demonstrated in cervical, ovarian, lung, liver, and breast carcinomas and was a predictor of increased malignancy and poor prognostic outcome [5, 6]. Although the importance of Sox-2 for ESC maintenance and induction of iPS cells has been clearly demonstrated [7, 8], it is unknown whether Sox-2 plays a role in regulating breast cancer stem cells (CSCs).

The CSC hypothesis postulates that neoplastic clones are maintained by a small subpopulation of cells that possess the capacity for self-renewal and differentiation potential, thus giving rise to cancer cells that comprise the tumor bulk [9, 10]. Furthermore, CSCs have been suggested to be the root cause of cancer recurrence and disease relapse due to their resistance to chemotherapy and radiotherapy [11]. CSCs were first identified in hematologic tumors [12] and were subsequently found in various solid malignancies including breast, brain, prostate, and lung cancers [13–16]. CSCs have also been discovered in various tumor-derived cell lines based on their expression, or lack thereof, of such surface markers as CD44, CD24, CD29, Lin, CD133, and Sca-1, and by their

Author contributions: Y.L.: conception, design and manuscript writing, and final approval of manuscript; R.A.R.: manuscript writing and final approval of manuscript; J.Y. and D.L.: collection of data and data analysis; C.C., Y.L., T.-H.C., R.X., and D.M.: collection of data. J.Y. and D.L. contributed equally to this article.

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ability to form spheres in nonadherent three-dimensional cultures in vitro [13, 16].

Another useful approach for identifying CSCs, particularly in the absence of suitable surface marker expression, uses the phenomenon of stem cells' unique ability to efflux lipophilic fluorescent dyes, including Hoechst 33342 [17]. This efflux capacity was shown to correlate with expression of ATP-binding cassette (ABC) transporters and could be specifically inhibited with Ca++-channel blockers [18]. The dye-effluxing population was given the designation side population (SP) based on their low dye retention characteristic [17, 19]. The dye-efflux method has been used to successfully identify SP cells possessing stem cell-like properties from a variety of tissues including mammary gland, skin, brain, and liver [20–23]. Additionally, previous work in our laboratory has demonstrated that this method can be used to isolate SP cells from murine breast carcinoma and neuroblastoma cell lines [24]. Importantly, compared to Non-SP, SP cells exhibited increased expression of stem cell-associated markers and showed enhanced tumorigenicity and resistance to chemotherapy when transplanted into immune-competent mice [24]. Furthermore, these SP cells also possessed a markedly higher repopulation potential in vivo, when compared with non-SP cells [24].

In vivo, stem cells are concentrated in areas rich in blood vessels and stromal cells, in regions referred to as the "stem cell niche." This niche is thought to protect stem cells from apoptotic stimuli and to enable a proper balance between self-renewal and differentiation. In fact, it was suggested that CSCs in the tumor microenvironment (TME) reside in a niche that is critical for their maintenance, similar to normal stem cells [25]. This theory implies that disruption of the CSC niche, and thus stem cell maintenance, may provide an avenue of attack for eliminating CSCs [26].

Tumor-associated macrophages (TAMs) constitute a major cell population in the breast TME [27]. Importantly, it has been suggested that macrophages secrete growth and other factors that permeate the breast stem cell niche to promote survival and self-renewal of stem cells [28]. Intriguingly, Ohno et al. showed that TAMs have varying responses to regulatory signals, depending on the area of the TME in which the signaling occurs [29]. Additionally, Pollard and coworker suggested that TAMs may be educated to carry out specific functions in support of maintaining CSC activities within perivascular and hypoxia/necrotic areas in the TME [30]. However, the precise role of TAMs in influencing CSC niches in vivo and the molecular mechanisms underlying TAM/CSC interactions remain unclear.

Therefore, in this study, we critically investigated whether TAMs regulate breast CSC maintenance. To this end, we identified a novel epidermal growth factor receptor (EGFR)/signal transducers and activators of transcription 3 (Stat3)/Sox-2 paracrine signaling pathway between macrophages and breast cancer cells that is required for upregulation of Sox-2 in tumor cells and maintenance of CSC phenotypes. Importantly, we show here that this crosstalk was effectively blocked by inhibition of either EGFR tyrosine kinase activity or Stat3 activation using the small molecule inhibitors AG1478 or CDDO-Im, respectively. To our knowledge, this is the first report identifying a mechanism by which TAMs regulate expression of Sox-2 in breast CSCs that establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for CSC-directed cancer therapy.

Materials and Methods

Animal Cell Lines and Reagents

Female BALB/c mice, 6–8 weeks of age, were purchased from the Scripps Research Institute Rodent Breeding Facility (La Jolla, CA). All animal experiments were performed according to National Institutes of Health guidelines. The 4T1 and 4T07 mammary carcinoma cell lines were generously provided by Dr. Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore). The RAW 264.7 macrophage cell line was purchased from ATCC (Manassas, VA). Our animal protocol was approved by The Scripps Research Institute Institutional Animal Care and Use Committee. AG1478, HOECHST 33342, and Verapamil hydrochloride were purchased from Sigma, Inc. (Sigma, St Louis, MO, http://www.sigmaaldrich.com). CDDO-Im was a gift from Dr. Michael Sporn (Dartmouth Medical School, Hanover, NH). Recombinant epidermal growth factor (EGF) was purchased from (BD Bioscience, Franklin Lakes, NJ, http://www.BD.com).

Flow Cytometry and Cell Sorting

We stained 1×10^6 tumor cells per milliliter with either 5 μg/ml HOECHST 33342 dye or HOECHST dye plus 100 mM Verapamil hydrochloride, to block dye efflux at 37°C for 30 minutes, as described previously [24]. These cells were then further stained with antibodies against Sca-1, c-Kit, ABCB1b, or ABCG2 (BD Pharmingen, San Diego, CA, http://www.BD.com) with appropriate isotype controls (BD Pharmingen). Cells were analyzed and sorted by an EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, CA, https://www.beckmancoulter.com). The HOECHST dye was excited at 350 nm, and its fluorescence measured at two wavelengths (450/20 nm band-pass filter and 675LP optical filter). Fluorescence-activated cell sorting data were analyzed with FlowJo software (Tree Star, Ashland, OR, http://www.treestar.com).

RT-PCR Analysis

Total RNA from three independently sorted 4T1 or 4T07 SP and non-SP cell samples was isolated using the RNAeasy Mini Kit (Qiagen, Valencia, CA, http://www.qiagen.com). RNA was then reverse transcribed applying the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and cDNA was analyzed by PCR. Alternatively, total RNA was analyzed using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen).

Western Blotting

Western blotting was performed with either total protein from cell lysate homogenates and cytoplasmic or nuclear extracts with polyclonal rabbit anti-mouse Sox-2, Oct-4, Stat3 or Nanog antibody, monoclonal rat anti-mouse E-cadherin antibody, and goat anti-mouse Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com). We also used polyclonal rabbit anti-mouse N-cadherin and beta catenin antibodies (Abcam, Cambridge, MA, http://www.abcam.com), or monoclonal rabbit anti-mouse phospho-Stat3 antibody (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com). Primary antibodies were detected with appropriate species-specific horseradish peroxidase-conjugated IgG antibodies (Santa Cruz Biotechnology).

Immunohistochemical Fluorescence Staining

4T1 or 4T07 cultured tumor cells were initially stained with HOECHST 33342 dye for 30 minutes at 37°C. The cells were then washed twice with cold phosphate-buffered saline (PBS) and stained with rabbit anti-mouse Sox-2 or Oct-4, rat anti-mouse EGFR (AbD Serotec, Raleigh, NC, http://www.abdserotec.com/), or rabbit anti-mouse Nanog (Santa Cruz Biotechnology) antibodies and detected with either goat anti-rabbit IgG Alexa 588, goat anti-rabbit IgG Alexa 568, or goat anti-rat IgG Alexa 488 secondary antibody (Life Sciences, Carlsbad, CA, http://www.lifesci.com). Macrophages were stained with a monoclonal rat anti-mouse F4/80 antibody (AbD Serotec) and detected by goat anti-rat IgG Alexa 568 secondary antibody (Life Sciences). Fluorescence images were captured with a Zeiss Axiovert 100 microscope (Carl Zeiss MicroImaging, Thornwood, NY, http://microscopy.zeiss.com).

Coculture Assay

4T1 breast carcinoma cells were cocultured with either RAW macrophage cells or macrophages derived from normal mouse spleen or

from breast tumor tissue. Briefly, for coculture without cell-cell contact, 3×10^5 macrophages were seeded in Boyden Transwell inserts (5-μm pores; Nunc, Rochester, NY, http://www.nuncbrand.com) permeable for soluble factors but not cells. Transwells containing macrophages were then inserted into a six-well plate and seeded with 8×10^5 4T1 tumor cells in each well. For coculture with cell-cell contact, 8×10^5/ml 4T1 tumor cells were mixed with 2×10^5/ml macrophages in a flask and then cultured together for different time points at 37°C, 5% CO_2, prior to analysis by flow cytometry.

In Vivo Macrophage Depletion

Chlodronate liposome nanoparticles were a gift from Roche Diagnostics GmbH (Roche, Mannheim, Germany, http://www.roche.com). Mononuclear phagocytes ingest the clodronate-containing liposomes and the liposomal bilayer is disrupted with phospholipase, resulting in intracellular release of clodronate and subsequent cell death [31]. A dose of 100 ml/10 g of mouse body weight of clodronate liposomes diluted in PBS was administered intravenously in BALB/c mice every week. The first injection was administered 2 days before the start of the experiment. Control mice were administered with either PBS as a control for unstimulated macrophages or liposomes in saline to control for any nonspecific effects of liposome administration. Macrophage depletion was maintained throughout the experimental period.

siRNA Gene Silencing

We used the siRNA gene Silencer system (Santa Cruz Biotechnology, Inc.) to perform the Sox-2 gene silencing in 4T07 tumor cells according to the manufacturer's protocol. Sox-2 knockdown was verified by RT-PCR or Western blot, as described above.

Migration Assay

Transwell migration assays were performed with 5×10^3 tumor cells seeded in Boyden Transwell chambers (8 μm pore size; Corning, Lowell, MA, http://www.corning.com). After 4-hour incubation at 37°C, cells that had migrated to the lower surface of the membrane were fixed with 1% paraformaldehyde, stained with 1% crystal violet, and counted.

Apoptosis Assay

Apoptosis was analyzed by flow cytometry using the Annexin V assay kit (eBioscience, San Diego, CA, http://www.ebioscience.com) according to the manufacturer's protocol. HOECHST 33342 dye was added 25 minutes before incubation with Annexin V to identify the SP or non-SP cell of 4T1 or 4T07 populations.

In Vivo Tumor Cell Challenge

Female age-matched BALB/c mice ($n = 4$ mice/group) were injected i.v. with either 1×10^4 4T07 wild-type or 4T07 Sox-2 knockdown breast carcinoma cells. All mice were sacrificed 25 days after tumor cell challenge.

Statistical Analysis

The statistical significance of different findings between experimental groups was determined by the Student's t test using Microsoft Excel (Microsoft, Redmond, WA, http://www.microsoft.com) or GraphPad Prism (GraphPad Software, LA Jolla, CA, http://www.graphpad.com) software. Results were considered significant if two-tailed p values were $< .05$.

RESULTS

TAM-Associated Increases in Breast Cancer Tumorigenicity Correlates with Increased Percentage of SP Cells In Vivo

Interactions between CSCs and cells within their niche in the TME appear to be important for their maintenance and function [26]. In order to assess the impact of TAMs on breast CSC maintenance and tumorigenicity in vivo, we first isolated 4T07 SP cells by flow cytometry and then injected 1×10^3 of these cells into the cleared fat pads of syngeneic BALB/c mice that were either depleted or not of macrophages by clodronate liposomes (Fig. 1A). The depletion of macrophages in both blood and tumor tissue was confirmed by flow cytometry (Fig. 1B). Analysis of mice 25 days after tumor cell implantation revealed significantly reduced primary tumor and lung weights in animals treated with clodronate liposomes, indicating reduced tumorigenicity and metastatic potential, respectively, in mice depleted of macrophages (Fig. 1C, 1D). Importantly, this reduction in tumorigenicity and metastasis in macrophage-depleted mice correlated with a significant decrease in the percentage of HOECHST 33342 dye-effluxing SP cells in primary tumors (Fig. 1E). Taken together, these results indicate that TAMs mediate maintenance of SP cell populations in vivo. Furthermore, these findings also imply that TAMs within the stem cell niche may play a critical role in CSC maintenance.

TAM Coculture Enriches the SP Population of 4TO7 Tumor Cells In Vitro

To further elucidate the mechanism of TAM-mediated regulation of breast CSCs, we performed a series of ex vivo experiments involving coculture of murine breast cancer cells with the murine RAW 264.7 macrophage cell line. This macrophage cell line was originally established from a tumor induced by Abelson murine leukemia virus [32] and we showed that these macrophages possess an M2/TAM phenotype characterized by expression of $CD206^{high}$, $IL\text{-}10^{high}$, $CD86^{low}$, and Major histocompatibility complex Class II^{low} (supporting information Fig. S1). Importantly, this phenotype was enhanced by either cocultured with tumor cell or tumor cell conditional medium with increasing release of Th2 cytokines and growth factors, which suppressed antitumor immunity and enhanced tumor growth (supporting information Fig. S2). We first analyzed the effects of RAW and tumor cell coculture on the SPs of 4TO7 tumor cells by the HOECHST 33342 dye exclusion assay. Flow cytometry analysis revealed that coculture of tumor cells with RAW macrophages resulted in a 2.8-fold increase in percentage of SP cells (Fig. 2A), which correlated with a marked increase in expression of Sca-1 and AbcG2 on the surface of these cells (Fig. 2B). At the mRNA and protein levels, macrophage coculture resulted in increased mRNA and protein expression of Sox-2, Oct-4, and Nanog by SP cells (Fig. 2C, 2D, respectively). Further analysis of the expression of Sox-2, Oct-4, and Nanog in primary tumors by immunohistochemistry revealed that these key transcription factors were expressed by tumor cells proximal to TAMs (Fig. 2E, 2F). Together, these results suggest that TAMs actively communicate with tumor cells to enhance their expression of Sox-2 and other key regulators of CSC phenotypes. These findings further imply that TAMs regulate breast CSCs by mediating their expression of Sox-2 through paracrine signaling within the stem cell niche.

The Sox-2 Transcription Factor Regulates the Tumorigenicity of SP Cells

Transcriptional regulation plays a key role in maintenance of CSC properties [33–35] and the Sox-2 transcription factor was reported to be important for regulating ESCs [33, 36]. Therefore, we postulated that the Sox-2 signaling pathway might be involved in networks controlling breast CSC maintenance. To determine whether Sox-2 is important for maintenance of CSC properties in murine breast cancer cells, we

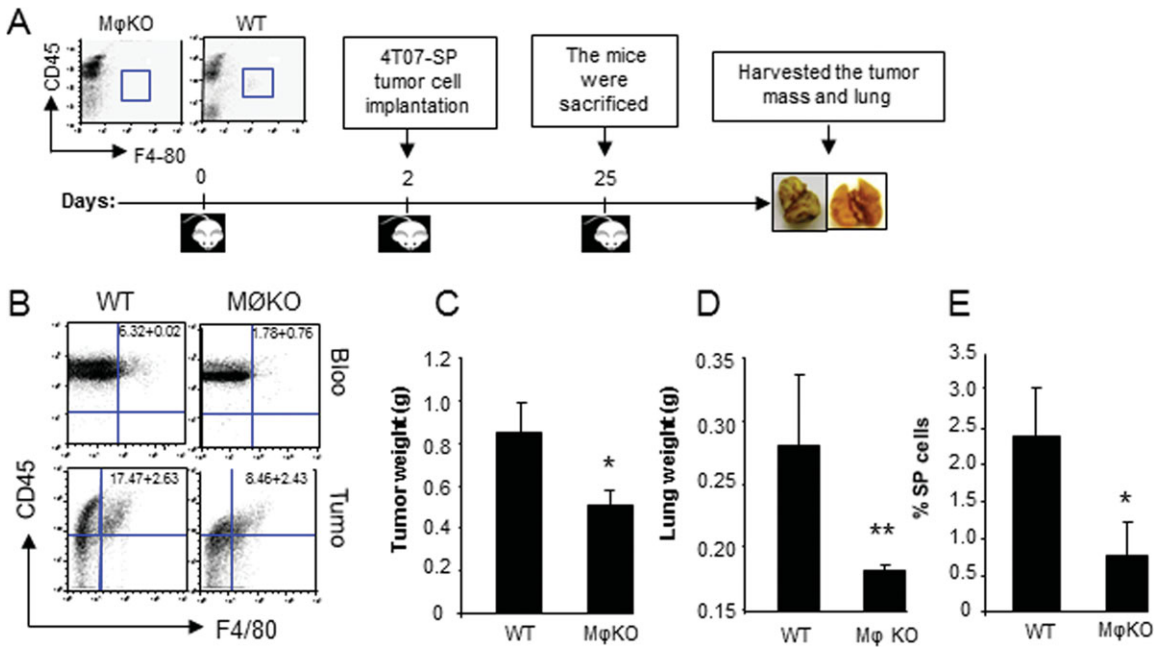

Figure 1. Tumor-associated macrophages mediate SP cell maintenance in vivo. **(A)**: 4TO7-SP cells were isolated by HOECHST 33342 dye staining and flow cytometry cell sorting. These SP cells (1×10^3) were then injected i.v. into BALB/c mice that had previously been depleted of macrophages by treatment with chlorinate liposome nanoparticles (M φ KO). Control animals were treated with saline and thus not depleted of macrophages (WT). ($n = 5$ mice/group), **(B)** Macrophage populations ($CD45^+/F4/80^+$) in blood and primary tumors of WT or M φ KO mice were measured by flow cytometry. Mice were sacrificed 25 days after SP cell challenge and tumor **(C)** and lung **(D)** weights were measured. Data represent means ± SEM. **(E)**: The percentages of SP cells in primary tumors from WT and M φ KO mice were measured by HOECHST 33342 dye staining and flow cytometry. Data represent means ± SEM. *, $p < .05$; **, $p < .005$. One of at least three independent experiments is shown. Abbreviations: SP, side population; WT, wild type.

used siRNA to silence Sox-2 gene expression in 4TO7 cells, which was confirmed by RT-PCR (Fig. 3A). Intriguingly, we found that Sox-2 silencing also resulted in decreased Oct-4 and Nanog mRNA expression (Fig. 3B). Furthermore, expression of Sca-1 and AbcG2 stem cell markers was also markedly suppressed in the SP population of 4TO7 cells treated with Sox-2 siRNA (Fig. 3C). In vivo, Sox-2 knockdown in 4TO7 cells markedly suppressed tumor growth (Fig. 3D), reduced tumor weights (Fig. 3E), and decreased lung metastasis, as indicated by decreased lung weight (Fig. 3F). Ex vivo transwell migration assays showed that Sox-2 silencing also suppressed tumor cell motility (Fig. 3G). Additionally, Sox-2 silencing in SP cells not only increased tumor cell apoptosis but also increased the sensitivity of these cells to mitoxantrone chemotherapy (Fig. 3H). Taken together, these data confirm that the transcription factor Sox-2 is important for the maintenance of CSC phenotypes in murine breast cancer cells.

TAM Activation of EGFRs on CSCs Results in Increased Sox-2 Expression by Tumor Cells Which Are Inhibited by EGF Neutralizing Antibody

We next aimed to identify the signaling molecules responsible for TAM-mediated upregulation of Sox-2 expression by breast cancer cells. TAMs are known to secrete many different growth factors and chemokines that promote tumor progression, including EGF [37]. Importantly in this regard, aberrant activation of the EGFR signaling pathway has been observed in many human cancers [38]. To determine whether soluble EGF released by TAMs mediates acquisition of CSC phenotypes by breast cancer cells, we examined expression of EGFR and phosphorylated EGFR (pEGFR) on 4T1 cells cocultured with RAW macrophages. Interestingly, analysis by flow cytometry showed overexpression of both EGFR and pEGFR by SP cells, compared with non-SP cells, in pure 4T1 cultures (Fig. 4A). Moreover, this expression of EGFR and pEGFR by SP cells was further enhanced by coculture with RAW macrophages, and correlated with an increase in percentage of SP cells, compared with tumor cells cultured alone (Fig. 4D). Similarly, culture of tumor cells with recombinant mouse EGF (mEGF) also induced an increase in the percentage of SP cells and a concordant increase in EGFR and pEGFR expression by these cells (Fig. 4E). Importantly, mEGF treatment resulted in increased expression of Sox-2 mRNA and protein by 4T1 tumor cells (Fig. 4B). This increase in Sox-2 expression also correlated with increased tumor cell migration upon mEGF stimulation (Fig. 4C). Together, these results demonstrate that TAM/tumor cell crosstalk via EGF/EGFR functions as an upstream activator of Sox-2 expression in murine breast cancer cells.

It was well known that TAMs release many kinds of cytokines and growth factors, including EGF, that promote tumor progress [39]. To determine whether EGF released by TAMs mediated maintenance of a CSC-like phenotype on SP of 4T1 breast cancer cell, we performed an experiment to detect expression of Sca-1 and ABCG2 on SP cells isolated from 4T1 tumor cells which were cocultured with TAMs after treatment with EGF neutralizing antibody. These results clearly indicated that expression of both Sca-1 and ABCG2 was downregulated by treatment of TAMs with EGF neutralizing antibody. The SP population of 4T1 cells was shown to be inhibited as well (Fig. 4F). Moreover, analysis of protein expression by 4T1 cells that had been cocultured, with either fresh isolated TAMs or RAW cells, demonstrated that expression of Stat3, pStat3, or Sox-2 was downregulated after treatment with EGF neutralizing antibody (Fig. 4G). Furthermore, migration of 4T1 cells was suppressed by treatment with EGF neutralizing antibody (Fig. 4H). Together, these data show that TAMs mediate effects that promote maintenance of SP CSC-like phenotypes supported by EGF release.

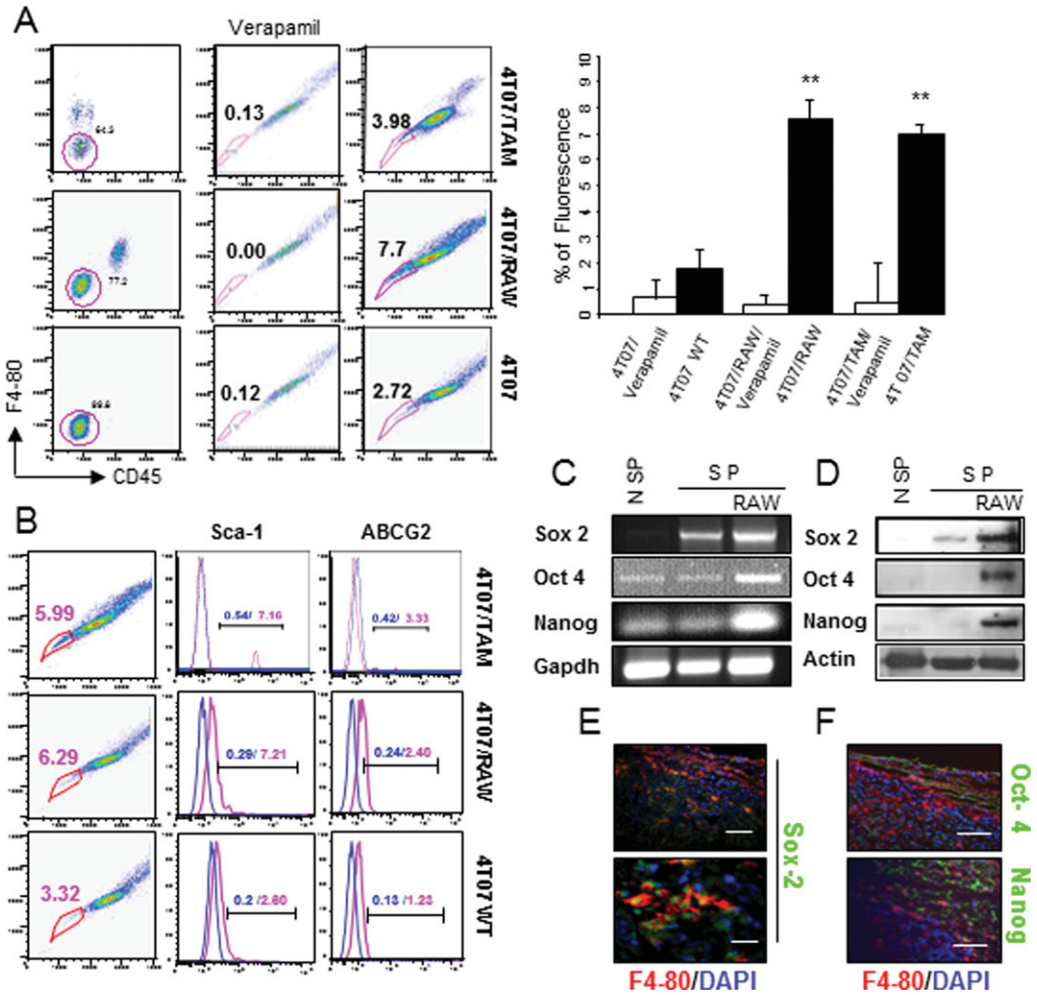

Figure 2. Tumor-associated macrophages enrich SP cells and enhance expression of Sox-2, Oct-4, and Nanog in breast cancer cells. **(A)**: The SP of 4T07 breast cancer cells was obtained by Hoechst staining and flow cytometry after 96 hours of coculture with either TAM derived from 4T07 tumor tissue or RAW macrophages. One of at least five independent experiments is shown. **(B)**: The expression of Sca-1 and ABCG2 was also detected in this same population. **(C)**: Expression of Sox-2, Oct 4, and Nanog was determined by RT-PCR and **(D)** Western blot. One of three independent experiments is shown. **(E, F)**: Expression of Sox-2, Oct 4, and Nanog in 4T07 tumor tissue was confirmed by immunofluorescence histology staining. Data represent means ± SEM. **, $p<0.005$, One of at least three independent experiments is shown. Scale bars = 100 μm; 150 μm on lower panel of (E). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; SP, side population; WT, wild type.

The EGFR Inhibitor AG1478 Blocks TAM-Mediated Upregulation of Sox-2 in Breast Cancer Cells

The requirement of EGFR activation for TAM/CSCs crosstalk and upregulation of Sox-2 expression by tumor cells was confirmed by blocking EGFR kinase activity with the inhibitor, AG1478. Treatment of 4T1 cells with AG1478 effectively blocked mEGF-induced upregulation of Sox-2 at both the mRNA and protein levels (Fig. 5A). Importantly, the increase in percentage of SP cells and expression of EGFR and pEGFR observed when 4T1 cells were cultured with RAW macrophage conditioned medium (MCM) was also completely blocked by AG1478 (Fig. 5B). Furthermore, analysis of mRNA and protein from these cells showed that AG1478 inhibited MCM-induced upregulation of both Sox-2 mRNA and protein expression (Fig. 5C). The inhibition of Sox-2 and EGFR expression by AG1478 was also confirmed by immunohistochemistry in 4T1 cells cocultured with either RAW macrophages or with RAW MCM (Fig. 5D, 5E, respectively). Together, these data show that TAM-mediated upregulation of Sox-2 expression in breast cancer cells is dependent on EGF/EGFR paracrine signaling.

EGFR-Mediated Upregulation of Sox-2 in Breast Cancer Cells Is Stat-3 Dependent

EGF is a known ligand for the Stat3 signaling pathway [40]. Upon binding of EGF to EGFR, Stat3 is phosphorylated which allows dimerization and translocation of the Stat3 protein into the nucleus where it activates transcription of downstream target genes [40]. Intriguingly, a recent study by Foshay et al. showed that Stat3 signaling could promote development of neural stem cells through regulation of Sox-2 expression [41]. Therefore, we hypothesized here that TAMs might use a similar paracrine signaling pathway via EGFR/Stat3 to regulate Sox-2 expression in breast cancer cells. Indeed, treatment of 4T1 cells with EGF induced phosphorylation of Stat3 and nuclear accumulation of Stat3 in a dose-dependent manner (Fig. 6A). In contrast, treatment of tumor cells with the Stat3 inhibitor, CDDO-Im, blocked EGF-induced phosphorylation of Stat3 (Fig. 6B). Critically, EGF treatment significantly increased the percentage of SP cells in 4T1 tumor cell cultures, and this increase was also effectively blocked by CDDO-Im (Fig. 6C). Furthermore, RT-PCR and Western blot analysis indicated that treatment of 4T1 cells

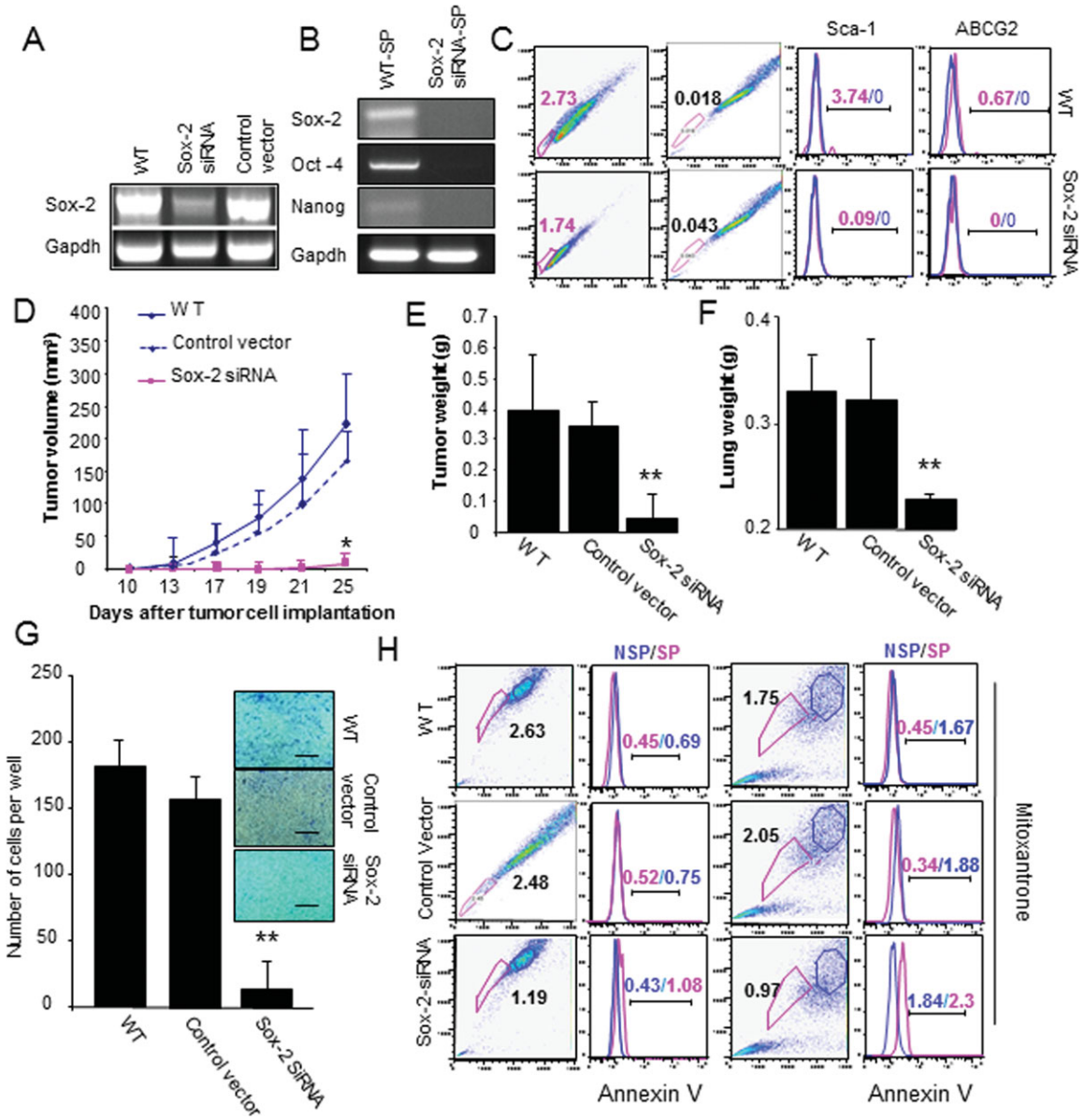

Figure 3. Transcription factor Sox-2 regulates maintenance of cancer stem cell-like properties of SP cells. Sox-2 gene expression in 4TO7 cells was silenced by siRNA. **(A)**: Downregulation of Sox-2 was confirmed by RT-PCR. **(B)**: Gene expression of Sox-2, Oct-4, and Nanog by SP cells was determined by RT-PCR after Sox-2 silencing. **(C)**: Expression of surface makers Sca-1 and ABCG2 was assessed by flow cytometry in 4TO7 SP cells after Sox-2 silencing. One of at least three independent experiments is shown. **(D)**: BALB/c mice were challenged with 4TO7-SP WT or 4TO7-SP cells subjected to Sox-2 silencing (Sox-2 siRNA), and tumor volumes were measured every 3–4 days. ($n = 5$ mice/group). Twenty-five days after tumor cell challenge, tumor **(E),** and lung **(F)** weights were measured. One of three independent experiments is shown. **(G)**: Migration assays were performed on 4TO7 WT or Sox-2 siRNA-treated SP cells using Boyden transwell chambers ($n = 3$ wells/group). **(H)**: Effects of Sox-2 silencing on apoptosis of non-SP and SP cells were determined by Annexin V staining and flow cytometry. Additionally, the sensitivity of SP and non-SP cells to mitoxantrone chemotherapy was also assessed after Sox-2 knockdown. Data represent means ± SEM. **, $p<0.005$. One of three independent experiments is shown. Scale bars = 500 mm. Abbreviations: SP, side population; WT, wild type.

with CDDO-Im also blocked EGF-induced upregulation of Sox-2 mRNA and protein expression (Fig. 6D, 6E). Additionally, partial inhibitions of Oct-4 and Nanog mRNA and protein expression were also observed with CDDO-Im treatment (Fig. 6D, 6E).

Since we found that CSC phenotypes can be induced by MCM and blocked by inhibiting EGFR tyrosine kinase activity via AG1478, we next determined whether these phenotypes could also be blocked through Stat3 inhibition by CDDO-Im. To this end, we treated 4T1 cells with EGF or IL-6 in combination with CDDO-Im. Western blot analysis revealed that only EGF treatment resulted in an increase in nuclear Stat3 and Sox-2 protein expression and was inhibited by CDDO-Im (Fig. 6F). Interestingly, EGF treatment also induced a decrease in E-cadherin and an increase in N-cadherin expression in 4T1 cells (Fig. 6F). This observation is significant since changes in cadherin expression have been linked to dedifferentiation of breast cancer cells and are associated with acquisition of CSC phenotypes [42]. Similar to EGF, incubation of breast cancer cells with MCM also resulted in nuclear accumulation of Sox-2 protein (Fig. 6G). Additionally, nuclear accumulation of β-catenin, and a

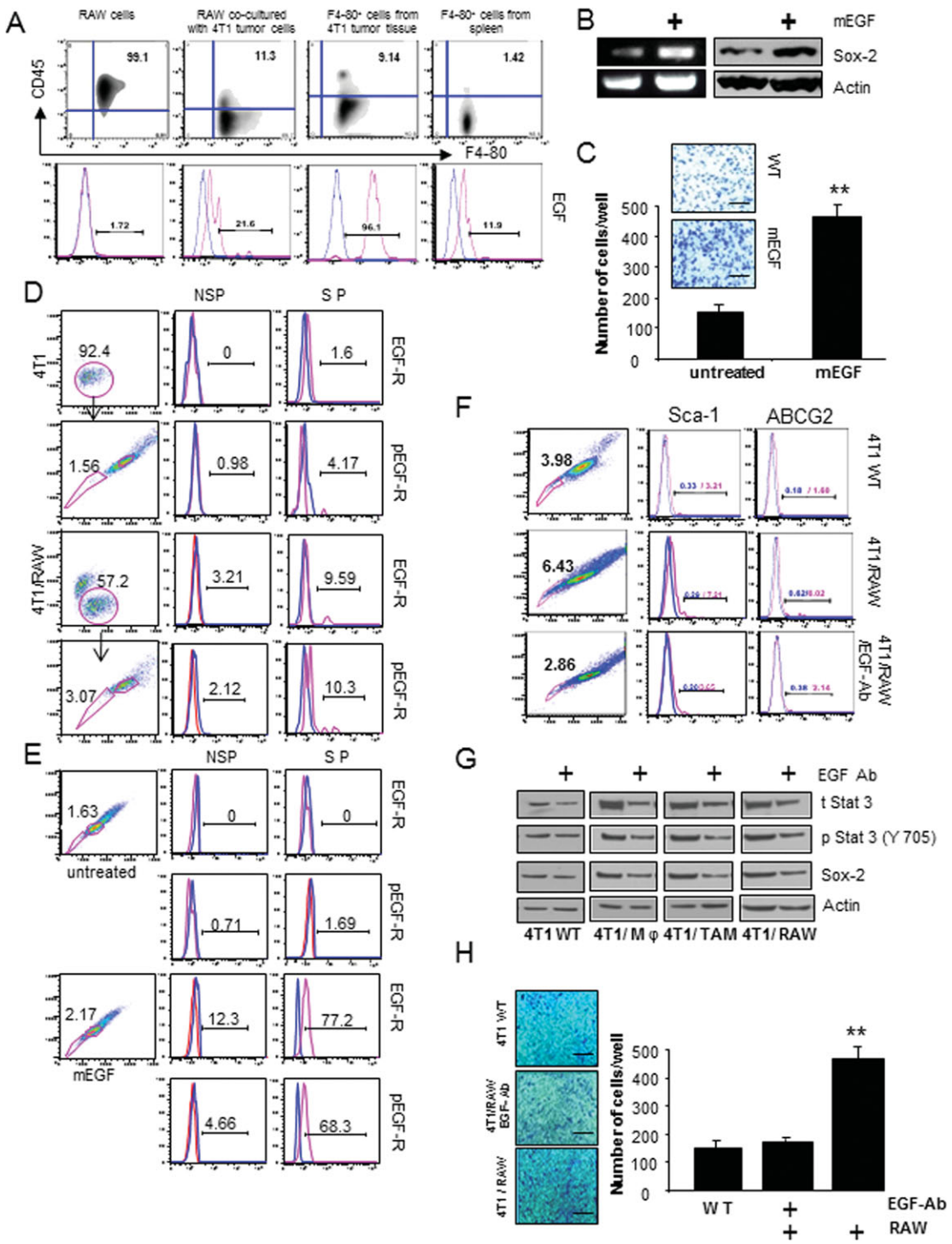

Figure 4. TAMs and EGF induce overexpression of EGFR and pEGFR on SP cells that correlates with increased Sox-2 expression and cell motility, which are inhibited by EGF neutralizing antibody. (**A**): The expression of EGF in F4-80+ cells from either 4T1 tumor tissue or normal spleen of BALB/c mice was determined by flow cytometry. EGF expression in RAW cells after cocultured with 4T1 tumor cells was also evaluated simultaneously. (**B**): Expression of Sox-2 at the mRNA (left panels) and protein levels (right panels) in 4T1 cells was assessed after treatment with mEGF. (**C**): EGF-induced migration of 4T1 cells as determined using Boyden transwell chambers. Expression of EGFR or phosphorylated EGFR (pEGFR) was detected by HOECHST 33342 dye and antibody staining, followed by flow cytometry analysis of 4T1 SP and non-SP cells after coculture with RAW macrophages (**D**) or recombinant mouse EGF (mEGF) (**E**). Data represent means ± SEM. (**F**): Expression of Sca-1 and ABCG2 in SP population of 4T1 cells was analyzed after coculture with RWA cells and treatment with EGF neutralizing antibody. (**G**): Either TAMs from 4T1 tumor tissue or RAW cells induced overexpression of Stat 3, pStat3, and Sox-2, which was inhibited by EGF neutralizing antibody in 4T1 cells. (**H**): The migration of 4T1 tumor cell after coculture with RAW cell was also inhibited by EGF-Ab. Data represent means ± SEM (three wells per group). , **, $p<0.005$ Scale bars = 500 mm. Abbreviations: mEGF, mouse epidermal growth factor; RAW, RAW264.7 cell line; SP, side population; TAM, tumor-associated macrophage; WT, wild type.

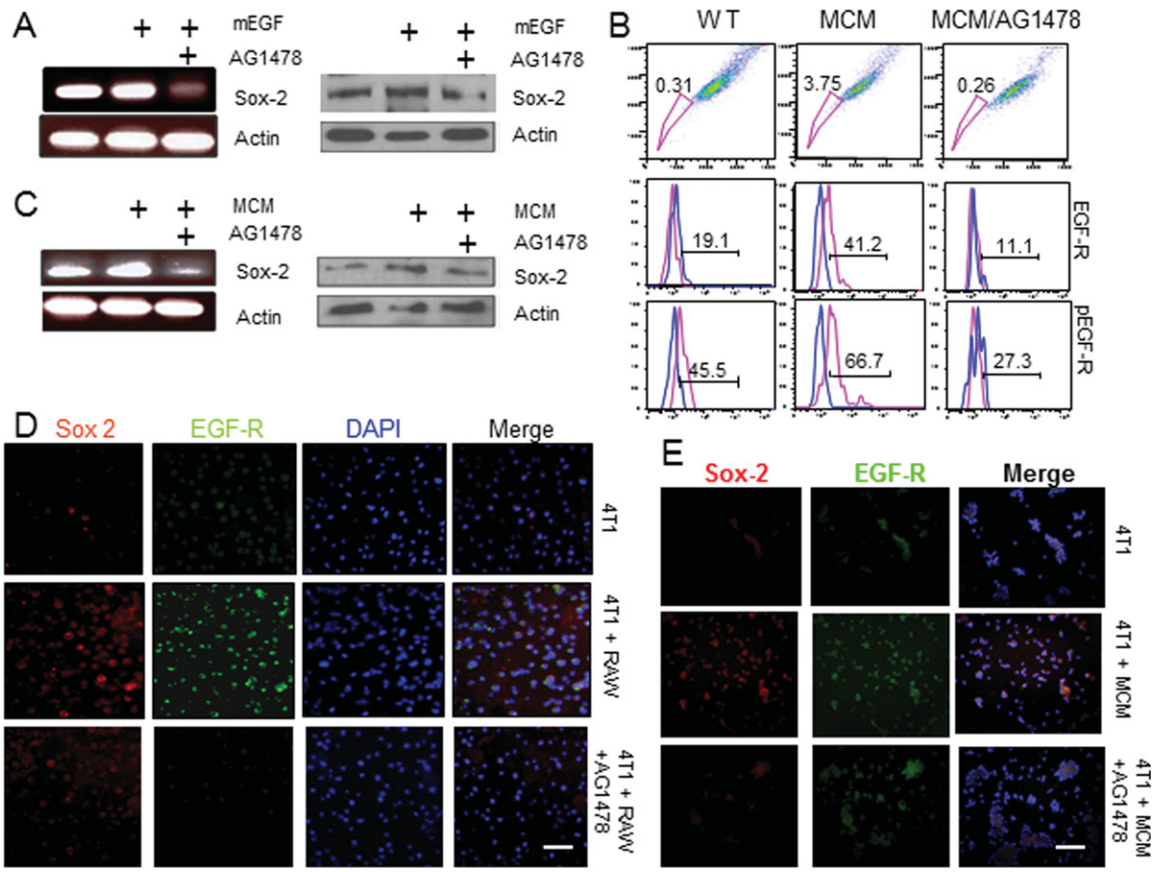

Figure 5. Macrophage-mediated effects on breast cancer cells are inhibited by EGFR inhibitor AG1478. **(A)**: EGF-induced overexpression of Sox-2 was found to be inhibited in 4T1 cells by AG1478, at both the mRNA (left panels) and protein levels (right panels). **(B)**: Culture of 4T1 cells with RAW MCM increased the percentage of side population cells and was correlated with increased EGFR and pEGFR expression as determined by HOECHST 33342, antibody staining, and flow cytometry. **(C)**: MCM-induced increases in Sox-2 expression are inhibited by AG1478 at both the mRNA (left panels) and protein levels (right panels). Inhibition of Sox-2 and EGFR expression after AG1478 treatment was confirmed by immunohistochemistry in 4T1 cells cocultured with either RAW macrophages **(D)** or MCM **(E)**. One of three independent experiments is shown. Scale bar = 100 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; mEGF, mouse epidermal growth factor; MCM, macrophage conditioned medium; RAW, RAW264.7 cell line; WT, wild type.

concomitant decrease in cytoplasmic β-catenin, protein expression was also observed in response to culture with MCM (Fig. 6G). Importantly, both upregulation of Sox-2 expression and nuclear translocation of β-catenin in response to MCM could be inhibited by treatment with CDDO-Im (Fig. 6G).

Because these changes in E- and N-cadherin expression and β-catenin localization are associated with increased cell motility, we investigated the effects of MCM on tumor cell motility. We found that transwell migration of both 4T1 and 4TO7 breast cancer cells was significantly increased when cultured with MCM (Fig. 6H). Importantly, this MCM-induced migration of tumor cells was significantly inhibited by CDDO-Im (Fig. 6I).

Discussion

It has been approximately 150 years since the idea of CSCs was first proposed. During this time, advances in stem cell biology have redefined the CSC hypothesis. This hypothesis now centers on the concept that a small fraction of tumor cells retains a self-renewing capacity that drives the tumorigenic potential. CSCs have been identified and isolated as a SP fraction from various human tumors on the basis of this self-renewing property [43]. Additionally, a better understanding of the TME has lead to the supposition that CSCs exist within a stem cell niche that regulates transcriptional networks important for the self-renewal capacity and tumorigenic potential of CSCs [25].

In this study, we provide further insight into the regulatory mechanisms that govern the stem cell niche, and its effects on Sox-2 expression in CSCs. We first confirmed the presence of CSC-like cells by identifying a HOECHST 33342 dye-effluxing SP in the 4T1 and 4T07 murine breast cancer cell lines (supporting information Fig. S2). In contrast to the dye-retaining non-SP, these SP cells possessed CSC phenotypes, including overexpression of Sox-2 and Oct-4 transcription factors and Sca-1 and AbcG2 cell surface markers. Association of these transcription factors and surface markers with stem cells is well established [44]. Additionally, SP cells isolated from murine breast cancer cell lines showed markedly increased tumorigenicity in vivo when implanted into syngeneic mice, in comparison to their non-SP counterparts. These findings are in agreement with previous work in our laboratory that identified SP cells in human breast cancer cell lines possessing CSC phenotypes, including increased tumorigenic potential in vivo [24]. Importantly, we found here that expression of the Sox-2 transcription factor was essential for

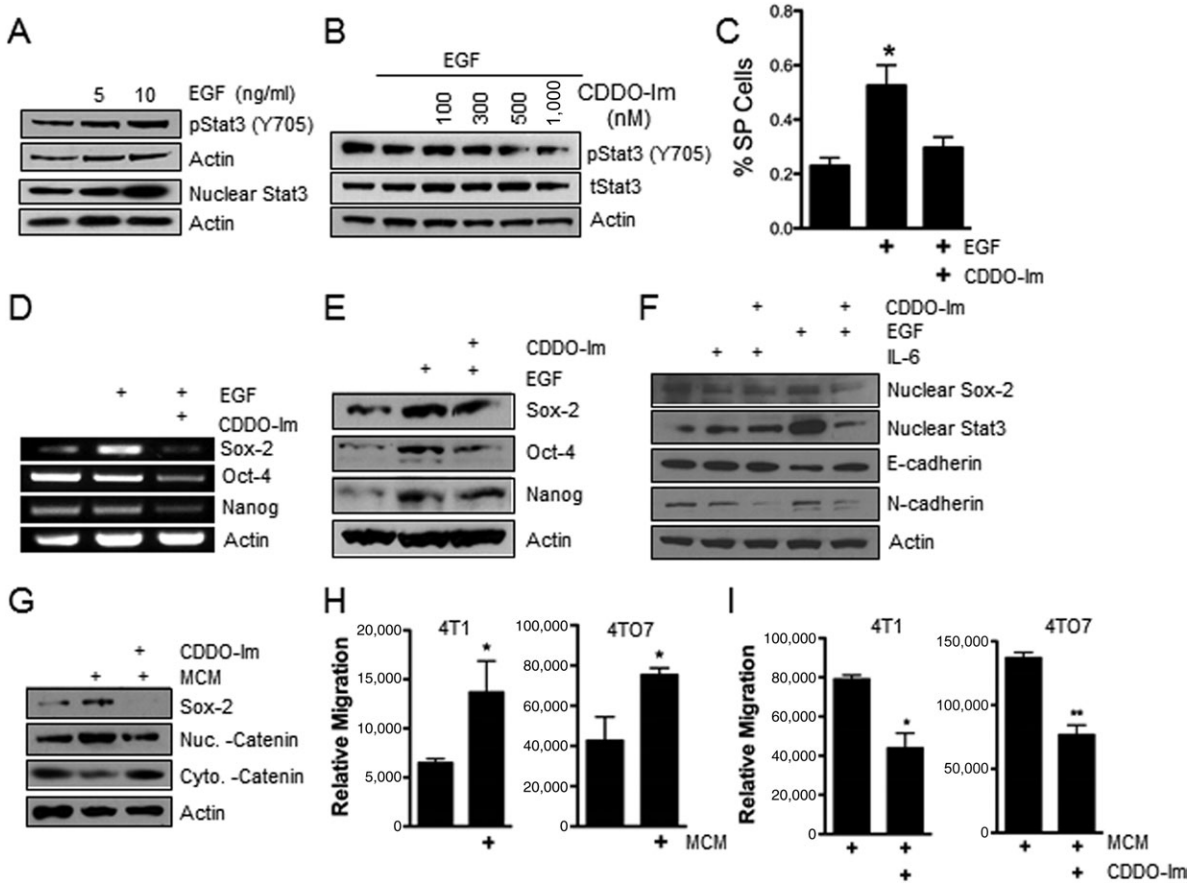

Figure 6. EGF-induced upregulation of SP cells is Stat3 dependent. **(A)**: Western blot analysis of cell lysates from 4T1 cells treated for 15 minutes with EGF at varying concentrations. **(B)**: Western blot analysis of cell lysates from 4TO7 cells treated for 10 minutes with EGF (20 ng/ml) plus CDDO-Im at varying concentrations. **(C)**: 4T1 cells treated with EGF, with or without CDDO-Im, were analyzed by HOECHST 33342 dye staining and flow cytometry to quantify the percentage of SP cells. Data represent means ± SEM. *, $p < .05$. **(D, E)**: Treatment of 4T1 cells with CDDO-Im (100 nM) suppresses EGF-induced upregulation of Sox-2, Oct-4, and Nanog mRNA (D) and protein (E) expression, as evaluated by RT-PCR and Western blotting, respectively. **(F)**: Western blotting of lysates from 4T1 cells, treated with either IL-6 or EGF in combination with CDDO-Im. **(G)**: Western blotting of lysates from 4T1 cells cultured in MCM, with or without CDDO-Im. **(H, I)**: Migration of 4T1 and 4TO7 breast cancer cells in response to incubation with MCM, either with or without CDDO-Im, was determined in Boyden transwell chambers and quantified with ImageJ software (three wells per group). *, $p < .05$; **, $p < .005$. One of three independent experiments is shown. Abbreviations: EGF, epidermal growth factor; MCM, macrophage conditioned medium; SP, side population.

maintenance of CSC properties in murine breast cancer cells. Thus, knockdown of Sox-2 in these cells by siRNA not only decreased expression of Sox-2 but also decreased expression of its partner genes Oct-4 and Nanog and Sca-1 and AbcG2 CSC surface markers by SP cells. Significantly, these reductions in CSC-associated gene expression and surface markers upon Sox-2 knockdown coincided with a concomitant decrease in the percentage of SP cells within the tumor cell population.

Increasing evidence emerging in the stem cell field suggests that acquired resistance to chemotherapy can be attributed to CSCs [45]. It is therefore intriguing that Sox-2 knockdown in SP cells resulted in decreased expression of ABC transporters which correlated with increased sensitivity of SP cells to mitoxantrone chemotherapy. These results may provide a potential avenue of attack for sensitizing chemo-resistant CSCs to chemotherapy through downregulation of Sox-2 expression in these cells.

Tissue niches play a critical role in the self-renewal and differentiation capacity of stem cells. For example, circulating hematopoietic stem cells have little function outside their tissue-specific niches and trafficking to specific tissue niches within the bone marrow is critical for maintaining normal hematopoiesis [46]. Considering that SP cells isolated from tumors share many characteristics with their normal stem cell counterparts, it is not implausible to predict that niches in which CSCs reside also play important roles in preserving their functions. Therefore, crosstalk between stromal and tumor cells within the CSC niche may regulate cytokine and growth factor expression levels to promote tumor cell migration or drug resistance.

In this regard, TAMs have been identified as a major cellular component of the breast TME and their trafficking from the circulation to the tumor stroma is a key phenomenon associated with breast cancer progression [27]. Additionally, TAMs are known to secrete various factors that promote tumor growth [47]. However, whether TAMs regulate CSC functions is not known. Due to their association with increased tumorigenicity and their physical proximity to tumor cells, we hypothesized that TAMs play a central role in CSC maintenance. Indeed, in support of our hypothesis, we found that depletion of macrophages in vivo suppressed the tumorigenicity of murine breast cancer cells implanted into syngeneic mice. Critically, suppressed tumor growth in these mice was associated with a decrease in the percentage of SP cells in their primary tumors. In contrast, ex vivo coculture of

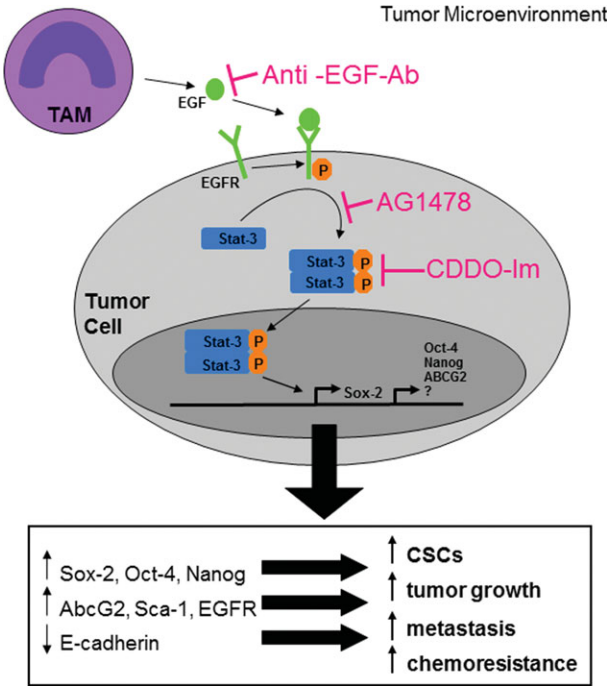

Figure 7. Schematic of interactions between macrophages and tumor cells in the breast tumor microenvironment. EGF released from TAMs binds to and activates EGFRs on tumor cells resulting in downstream phosphorylation of Stat3. Activation of Stat3 promotes transcription and expression of Sox-2 and other target genes involved in maintenance of CSC phenotypes. Acquisition of these phenotypes by tumor cells results in increased tumor growth, metastasis, and chemoresistance. Induction of such TAM-driven CSC phenotypes in tumor cells can be inhibited by anti-EGF neutralizing antibody, AG1478—a small molecule inhibitor of EGFR protein tyrosine kinase activity or CDDO-Im—a small molecule inhibitor of Stat3 phosphorylation and activation. Abbreviations: CSC, cancer stem cell; EGF, epidermal growth factor; TAM, tumor-associated macrophage.

such tumor cells with RAW macrophages lead to an increase in percentage of SP cells and correlated with increased expression of CSC-associated genes, including Sox-2. These results demonstrate that TAMs mediate Sox-2 expression in tumor cells and thus represent a stromal cell responsible for regulating and maintaining CSCs in the stem cell niche of the breast TME.

Even though the existence of crosstalk between CSCs and niche cells is well-established [48, 49], the cellular mechanisms mediating this crosstalk and the signaling molecules involved remain a complex web waiting to be untangled. To this end, we identified EGF as a key signaling molecule that mediates crosstalk between TAMs and tumor cells. We also showed that EGF enriches the percentage of SP cells in murine breast cancer cell cultures and increases their expression of Sox-2. Additionally, EGF also increased tumor cell motility. Critically, all these effects were effectively blocked by AG1478, an EGFR tyrosine kinase inhibitor, confirming the essential role of EGF in TAM/tumor cell crosstalk. These results are intriguing given that constitutive activation of EGFR is commonly observed in epithelial tumors and is a validated target for therapy of solid cancers, including breast cancer [38, 50].

Since EGF is a known ligand for the Stat3 signaling pathway, and Stat3 has recently been shown to mediate Sox-2 expression in neural stem cells [41, 51], we hypothesized that this transcription factor could be a downstream target of EGFR signaling that mediates CSC phenotypes in breast cancer cells. Importantly, phosphorylation of Stat3 at Y705 is critical for homodimerization and nuclear translocation of the protein, thus mediating its transcriptional activity [51]. Synthetic triterpenoids are a novel class of multifunctional inhibitors that have been shown to inhibit the growth of many solid tumors. In particular, an imidazole derivative of synthetic oleanane triterpenoid (CDDO-Im) has been shown to be a potent inhibitor of Stat3 phosphorylation at Y705 [52]. Additionally, we showed here that blocking Stat3 phosphorylation with CDDO-Im effectively inhibited EGF-induced increases in breast cancer SP cells and upregulation of Sox-2, Oct-4, and Nanog expression. Furthermore, CDDO-Im also blocked MCM- and EGF-induced decreases in E-cadherin and increases in N-cadherin expression as well as β-catenin nuclear localization. These results are significant since formation of CSCs has been linked to epithelial-to-mesenchymal transitions, which is characterized by loss of E-cadherin, a gain of N-cadherin expression, and nuclear translocation of β-catenin. In turn, these changes are associated with increased tumor cell motility and tumorigenicity. Importantly, the increase in tumor cell motility we observed upon culture with MCM was also inhibited by CDDO-Im. These results are of particular interest since CDDO-Im effectively blocked these TAM-induced CSC phenotypes at nM concentrations. Therefore, due to its potency, CDDO-Im may be a useful chemotherapeutic drug to be developed for cancer therapy targeting CSCs

Conclusions

As summarized schematically in Figure 7, our collective results demonstrate that TAMs play a key role in promoting maintenance of CSC phenotypes in murine breast cancer cells. Importantly, we have identified a novel mechanism of regulation that is achieved by paracrine EGF signaling between TAMs and tumor cells. We further demonstrated that this signaling involves activation of the EGFR/Stat3 signaling pathway and the downstream upregulation of the Sox-2 transcription factor. Critically, this TAM/tumor cell crosstalk required EGFR and Stat3 activity and could be blocked by inhibitors of either EGF or Stat3. To our knowledge, we are first to describe this unique interaction between TAMs and breast cancer cells via EGF/EGFR/Stat3 signaling that is critical for Sox-2 expression and breast CSC maintenance. Importantly, our report identifies a novel role for macrophages in breast CSC regulation and establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for CSC therapy.

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Disclosure of Potential Conflicts of Interest

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ORIGINAL ARTICLE

MicroRNA-19a-3p inhibits breast cancer progression and metastasis by inducing macrophage polarization through downregulated expression of *Fra-1* proto-oncogene

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One of the hallmarks of malignancy is the polarization of tumor-associated macrophages (TAMs) from a pro-immune (M1-like) phenotype to an immune-suppressive (M2-like) phenotype. However, the molecular basis of the process is still unclear. MicroRNA (miRNA) comprises a group of small, non-coding RNAs that are broadly expressed by a variety of organisms and are involved in cell behaviors such as suppression or promotion of tumorigenesis. Here, we demonstrate that miR-19a-3p, broadly conserved among vertebrates, was downregulated in RAW264.7 macrophage cells of the M2 phenotype in conditoned medium of 4T1 mouse breast tumor cells. This downregulation correlated with an increased expression of the *Fra-1* gene, which was reported to act as a pro-oncogene by supporting the invasion and progression of breast tumors. We found significant upregulation of miR-19a-3p in RAW264.7 macrophages after transfection with a miR-19a-3p mimic that resulted in a significant suppression of the expression of this gene. In addition, we could measure the activity of binding between miR-19a-3p and Fra-1 with a psiCHECK luciferase reporter system. Further, transfection of RAW264.7 macrophage cells with the miR-19a-3p mimic decreased the expression of the Fra-1 downstream genes *VEGF*, *STAT3* and *pSTAT3*. Most importantly, the capacity of 4T1 breast tumor cells to migrate and invade was impaired *in vivo* by the intratumoral injection of miR-19a-3p. Taken together, these findings indicate that miR-19a-3p is capable of downregulating the M2 phenotype in M2 macrophages and that the low expression of this miRNA has an important role in the upregulation of Fra-1 expression and induction of M2 macrophage polarization.

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Keywords: tumor-associated macrophages; tumor microenvironment; Fra-1; miR-19a-3p

INTRODUCTION

MicroRNAs (miRNAs) comprise a family of ~22-nucleotide-long RNA molecules. They are transcribed from various genetic regions and processed in the nuclei through the cytoplasm, becoming mature single-stranded miRNA molecules. It is well recognized that miRNAs regulate gene expression in eukaryotic cells.[1–3] They are expressed in various species and are involved in cellular processes by targeting the 3′ untranslated regions (UTRs) of target mRNAs. MiRNAs function as negative regulators of gene expression by increasing mRNA decay or by blocking the process of translation. Altered expression of the miRNA species is well established in human cancer cells,[4–6] which are involved in tumor development, progression and metastasis by targeting mRNAs of oncogenes or tumor suppressor genes. Moreover, the different types of cancer cells have revealed distinct miRNA expression profiles, indicating that analysis of miRNA expression patterns could aid in identifying miRNAs that regulate tumor progression.[7–10] Although miRNAs are becoming increasingly established as regulatory molecules in cancers, the roles of miRNA expression in tumor development and as potential markers for diagnosis, prognosis and pharmacogenomics remain to be established.

Tumor-associated macrophages(TAMs) coexist in tumors and function as an accomplice to promote tumor progression and metastasis, especially once programed and polarized into a proangiogenic/immune-suppressive(M2-like) phenotype by the tumor microenvironment (TME).[11] There is increasing evidence to show that TAMs accumulate mainly in the vascular, necrotic/hypoxic areas of tumors, where they presumably act to clean necrotic cell debris from their sites.[12] TAMs located in these sites of solid tumor respond to the hypoxia present with altered gene expression, leading to the development of a distinct pro-tumor phenotype. In this case, TAMs provide an ideal therapeutic target for blocking tumor progression and invasion after being re-programed and polarized to express a pro-immune (M1-like) phenotype.[13–15]

Fos-related antigen-1 (*Fra-1*) belongs to the AP1 family, which is overexpressed by many human and mouse epithelial tumor cells and is associated with metastasis to lymph nodes.[16] Actually, Fra-1 has been considered to have prognostic significance, particularly for human esophageal squamous cell carcinoma, and to be a regulator of the malignant phenotype of glioma cells.[17,18] We previously reported that *Fra-1* has a key role in the polarization of TAMs from the M1 to the M2 phenotype. In other words, expression of *Fra-1* could be upregulated in TAMs that responded to stimulation by TME, leading to an increased proportion of M2-like TAMs.[19,20] Recently, it was reported that Fra-1 is indeed a miRNA target that may control tumor invasion and migration.[21,22]

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Here, we identified the microRNA expression pattern in TAMs induced by stimuli from the TME in a mouse breast tumor model. Further, our study demonstrated an important normal mechanism by which miRNAs could induce the phenotype switch between TAMs and normal macrophages by regulating the expression of the *Fra-1* gene and the Fra-1/STAT3 signaling pathway. Together, these findings aided us in exploring the potential of therapy targets on miRNA capable of regulating the switching of the TAM phenotype, resulting in remodeling of the TME.

RESULTS

Tumor microenvironment changes miRNA expression patterns and enhances the expression of Fra-1 in TAMs

A search for potential miRNAs that target the 3′UTR of Fra-1 was based on the miRBase and TargetScan software prediction. The expression of miR-19a-3p was downregulated significantly in the mouse leukemia macrophage cell line RAW264.7 after culturing with mouse breast cancer cell line 4T1-conditioned medium of three candidates (miR-19a-3p, miR-29a and miR-503) and was compared with that in wild-type RAW264.7 cells (Figure 1a and b). As miR-19a-3p is broadly conserved among vertebrates, an investigation was conducted to determine whether its functions are similar to that of human macrophage cells. This was done by detecting the expression of miR-19a-3p in the human monocyte/macrophage cell line U937 cocultured with human breast cancer cell line MDA-MB-231-conditioned medium; the results were similar to those obtained in Figure 1c. To investigate the miRNA expression patterns in TAMs *in vivo* that were induced by TME, total RNA was collected from TAMs derived from mouse 4T1 breast cancer tissue, and the miRNA profile was determined using the miRNA qPCR technique. The data indicate that miR-19a-3p expression was significantly decreased in TAMs compared with that in normal macrophages from the spleen (Figure 1d). Simultaneously, we confirmed the expression of Fra-1 in RAW264.7 cells cocultured with 4T1-conditioned medium. The data reveal an upregulated *Fra-1* gene expression (Figure 1e). The same phenomenon was observed in the human macrophage U937 cell line (Figure 1f). Taken together, these results suggest that the 3′UTR of the *Fra-1* gene is a potential target of miR-19a-3p that can regulate *Fra-1* gene expression in TAMs within breast tumor TME.

MiR-19a-3p downregulates Fra-1 and its downstream pathway genes

The regulation of miRNA to the transcription factor and its governing downstream signaling pathway has been recognized to be critical for malignant tumor progress. Here, the opposite of the

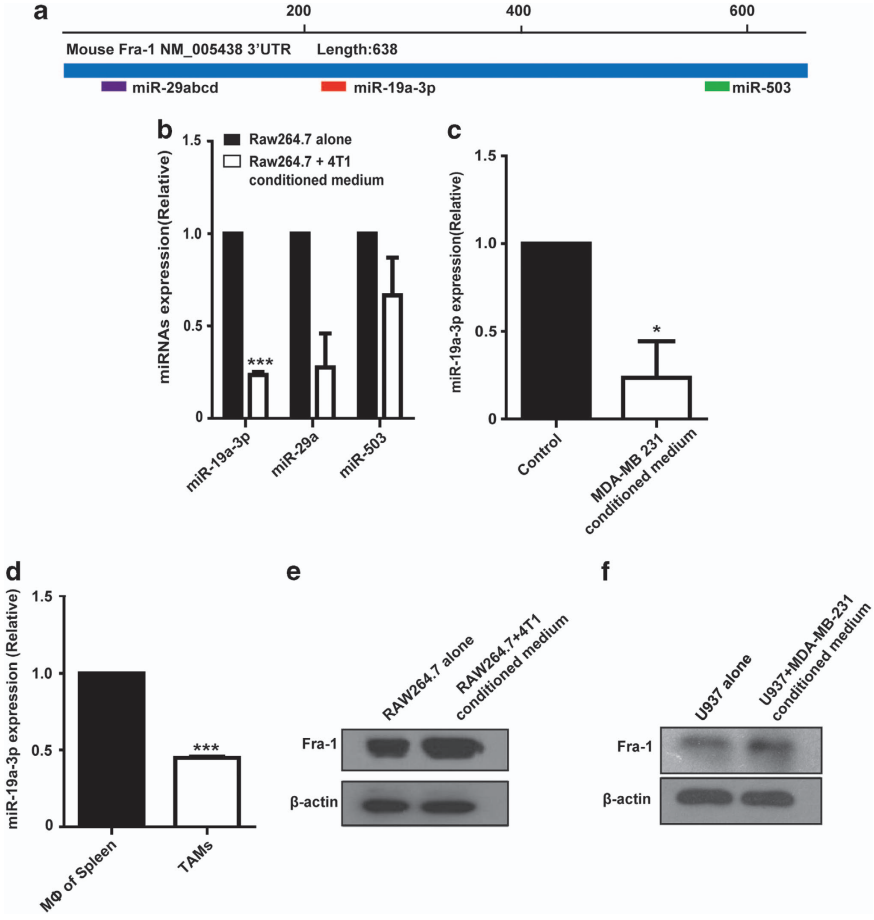

Figure 1. Tumor microenvironment changes miRNA expression patterns and enhances the Fra-1 expression of TAMs. (**a**) Prediction of Fra-1 3′UTR binding by miRNAs based on targetscan online software. (**b**) MiR-19a-3p, miR-29a and miR-503 expression by RAW264.7 cells, cocultured with 4T1-conditioned medium, as detected by real-time PCR ($n=3$ $^{***}P<0.001$). (**c**) Expression of miR-19a-3p of U937 cells cocultured with MDA-MB-231-conditioned medium was tested by real-time PCR. Data are shown as mean ± s.e.m from three independent experiments ($n=3$, $^{*}P<0.05$). (**d**) Total RNA was collected from TAMs and normal macrophages derived from mouse 4T1 breast cancer tissue and spleen, respectively, and miRNA expression was detected by real-time PCR. (**e**) The expression of Fra-1 was examined by western blot in RAW264.7 cells that were cocultured with 4T1cells-conditioned medium. (**f**) Expression of Fra-1 in human macrophage U937 cells cocultured with conditioned medium of MDA-MB-231 cells was examined by western blots, with β-actin used as the loading control.

relationship between Fra-1 and miR-19a-3p expression of TAMs was observed, implying that the 3′UTR of Fra-1 could be a potential target of miR-19a-3p. To confirm this, the miR-19a-3p mimic was synthesized and transfected into RAW264.7 cells. In this case, Fra-1 expression was significantly suppressed by the miR-19a-3p mimic within 24 h (Figure 2a and Supplementary Figure 1B), but not by miR-503 and miR-29a (Supplementary Figure 1A). The same result was also observed in primary mouse macrophages and human macrophage U937 cells (Figure 2b). Further, to confirm our prediction, we generated a reporter vector consisting of the luciferase coding sequence followed by the 3′-UTR of the Fra-1(Luc-Fra-1-3′-UTR) mutant, with the putative binding site used as a negative control (Figure 2c). These constructs were co-transfected into RAW264.7 cells, and luciferase activity was analyzed. These co-transfection experiments showed that miR-19a-3p decreased the luciferase activity of Luc-Fra-1-3′-UTR, but had only a minimal effect with the negative control plasmid (Figure 2d). To further explore whether miR-19a-3p could regulate the downstream STAT3 pathway of Fra-1 via inhibition of Fra-1 expression, the miR-19a-3p mimic and inhibitor were synthesized and then transfected into RAW264.7 cells. In this case, the expressions of pSTAT3 and STAT3 and of their target gene *VEGF-A* were either suppressed (Figure 2e) or promoted (Figure 2f). Data from immunofluorescence experiments reveal the same trend (Figure 2g). Together, these findings indicate that miR-19a-3p can bind to the 3′-UTR of Fra-1 and regulate the expression of Fra-1 and the activity of its downstream pathway. Dual-luciferase signal reporter array showed that miR-19a-3p also downregulates other signaling pathways that promote M2 polarization of macrophages, especially the glucocorticoid and NFAT pathway, and upregulates the estrogen pathway that inhibits the M2 polarization of TAMs (Supplementary Figure 1c).

Downregulated miR-19a-3p promotes the M2 phenotype of TAMs by releasing control of Fra-1/STAT3 pathway activity

Our previous study indicated that overexpression of Fra-1 in TAMs promotes the invasion and progression of breast cancer cells.[19] We determined whether inhibition of Fra-1 by miR-19a-3p in RAW264.7 cells could indeed impair the invasion and migration capacity of breast tumor cells in the TME. To this end, three mouse breast tumor cell lines, 4T1, 4T07 and EMT6, were used as follows. Transwell assays indicated that the invasion capacity of these three tumor cell lines was significantly suppressed when cocultured with RAW264.7 cells after being transfected with miR-19a-3p (Figure 3a). Meanwhile, the wound healing experiment clearly showed that the migration property of 4T1 and EMT6, except 4T07, was also attenuated (Figure 3b), indicating that transfection of miR-19a-3p into TAMs inhibits any effect that it may have on the metastatic properties of breast tumor cells.

Polarization of M1 to the M2 phenotype is an important transformation of macrophages in the TME. To prove that the regulation of miR-19a-3p may support the maintenance of the M2 phenotype of TAMs in the breast tumor TME, an analysis of M1 or M2 marker expression was performed in mouse primary macrophages or RAW264.7 cells transfected with either the miR-19a-3p mimic or its miR-negative control using real-time PCR or flow cytometry. Results from these experiments showed that the expression of M2 markers Arg1 and CD206 was significantly decreased. Thus, reverse M1 marker Mcp1 and Nos2 expressions were increased in macrophages that were transfected with the miR-19a-3p mimic when compared with negative controls (Figures 4a and b). Further, experiments were designed to test whether cytokines secreted by macrophages could be changed by overexpressing miR-19a-3p in RAW264.7 cells. To this end, supernatants were collected from RAW264.7 cells transfected with either the miR-19a-3p mimic or its miR-negative control and used to detect cytokine levels by means of a mouse cytokine array panel. In fact, M2-type cytokines such as interleukin (IL)-4, IL-6, IL-10 and IL-13 were significantly decreased, whereas M1-type cytokines CXCL10 and Mcp1 were increased, when miR-19a-3p was overexpressed in RAW264.7 cells (Figure 4c). These observations suggest that miR-19a-3p can help transform the M2 phenotype into RAW264.7 cells by regulating the expression of the Fra-1/STAT3 pathway.

MiR-19a-3p inhibits metastasis of 4T1 breast cancer cells by suppressing M2 macrophage function *in vivo*

After identifying the function of miR-19a-3p in transforming the M2–M1 phenotype of TAMs *in vitro*, we explored its function *in vivo*, particularly as it aids in the invasion and migration of breast cancer cells. For this purpose, 4T1-luciferase-labeled cells were injected into Balb/c mice. Thereafter, these mice xenografted with 4T1 cells were injected twice a week intratumorally with miR-19a-3p agomir, a chemically modified miRNA mimic, or with the negative control. Tumor mass and lungs from these mice were harvested after 3 weeks (Figure 5a) and further tested for the presence of CD206-positive cells by immunohistochemistry and immunofluorescence staining. The number of CD206-positive cells in tumor tissue was seen to be significantly decreased after injection with miR-19a-3p agomir (Figures 5b and c). Consistent with these results obtained by immunohistochemical staining, a decrease was observed in the number of F4/80 + CD206 + cells in tumors injected with miR-19a-3p agomir compared with negative controls, as found by flow cytometry (Figure 5d).

To determine whether miR-19a-3p inhibited the growth and metastasis of 4T1 breast tumor by suppressing macrophages with the M2 phenotype, the tumor volume and lung metastasis in Balb/c mice were ascertained by live image and hematoxylin and eosin staining. We found that injecting miR-19a-3p agomir into the 4T1 tumor model resulted in a significantly attenuated metastatic capacity for tumor cells, but not for original tumor growth (Figures 5e and f). These data suggest that miR-19a-3p mainly regulates TAMs to inhibit metastases of 4T1 tumor cells. Together, our data indicate that miR-19a-3p is downregulated in macrophages with the M2 phenotype. The low expression of miR-19a-3p apparently has an important role in the upregulation of Fra-1 expression and induction in M2 macrophage polarization, which may contribute considerably to facilitating the metastasis and progression of breast cancer.

IL-6 activates the M2 polarization-related signal pathway by inhibiting miR-19a-3p expression of TAMs

IL-6 has been reported to be a regulator in the promotion of M2 macrophages. To better understand whether IL-6 in the TME is involved in the downregulation of miR-19a-3p in TAMs, an effort was made to detect miR-19a-3p expression by real-time PCR with IL-6. The results showed that miR-19a-3p was indeed inhibited after 6 h of stimulation. Meanwhile, we also found that the mRNA of Fra-1 expression was also promoted by TAMs cultured in medium mixed with IL-6 (Figure 6a). Combined with these results obtained previously, here we drew out a schema summarizing the mechanism by which TME regulates the phenotype of TAMs by suppressing miR-19a-3p expression. We found that, specifically, IL-6 produced by tumor cells inhibits miR-19a-3p expression of TAMs; subsequently, signal pathways such as AP1/STAT3, glucocorticoid and nuclear factor of activated T cells (NFAT) are activated; and TAMs are polarized to the M2 phenotype to promote the progression and metastasis of tumor cells (Figure 6b).

DISCUSSION

In contrast to normal macrophages, there is increasing evidence that tumor-associated macrophages are capable of recruiting to the tumor site, increasing tumor angiogenesis and tumor cell

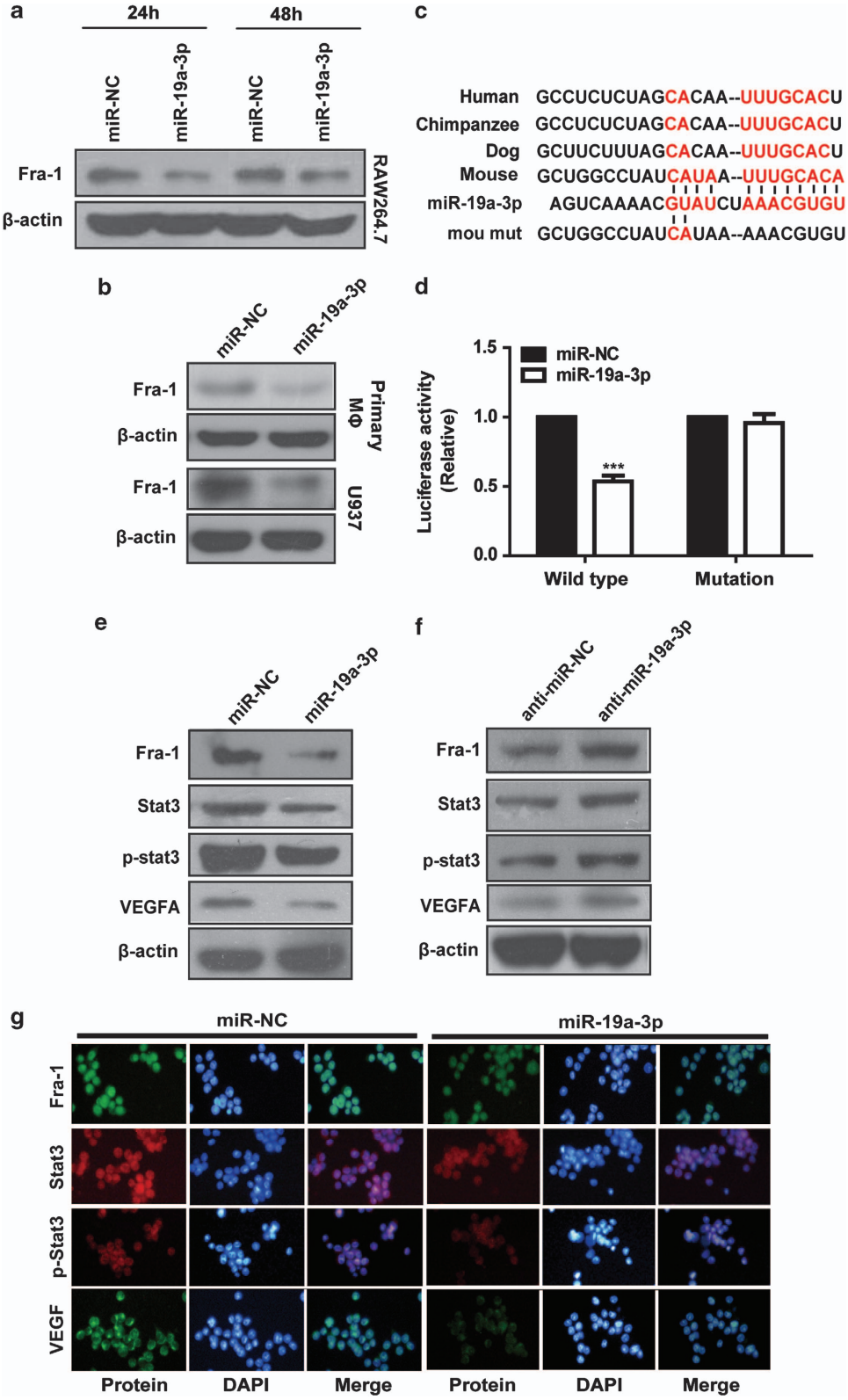

Figure 2. Low-level expression of miR-19a-3p upregulates the expression of Fra-1 and Fra-1/Stat 3 signaling pathways in macrophages. (**a**) Expression of Fra-1 in RAW264.7 cells that were treated with the miR-19a-3p mimic for 24 or 48 h. (**b**) Fra-1 expression in primary mouse macrophages and human U937 macrophages. (**c**) Predicted miR-19a-3p binding sites in the 3′UTR of Fra-1 in different species and a mutant site cloned by the psiCHECK2 dual-luciferase detection vector. (**d**) HEK293T cells were co-transfected with the miR-19a-3p mimic and psiCHECK2 reporter plasmid that was inserted with the Fra-1 3′ UTR. Luciferase activity was measured 24 h after transfection. The y-axis represents relative luciferase activity. Data are shown as mean ± s.e.m. from three independent experiments ($n = 3$, $^{***}P < 0.001$). (**e**,**f**) The MiR-19a-3p mimic and inhibitor were synthesized and transfected into RAW264.7 cells, and expressions of pSTAT3, STAT3 and VEGF-A were detected by western blots. (**g**) Expressions of Fra-1, STAT3 and pSTAT3 were detected by immunofluorescence in RAW264.7 cells that had been transfected with the miR-19a-3p mimic or miR-negative control.

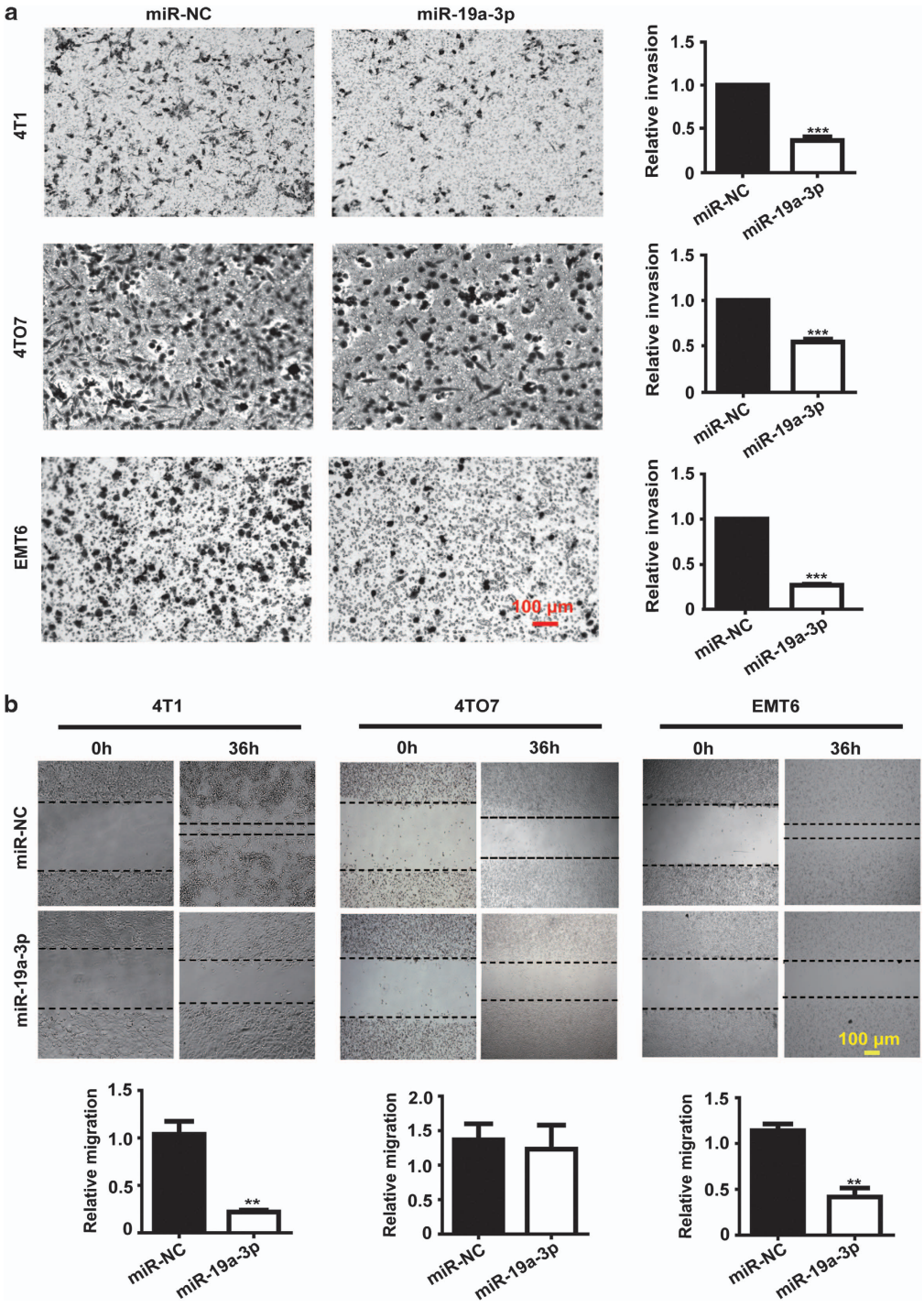

Figure 3. Invasion and migration capacities of RAW264.7 cells are inhibited by miR-19a-3p by suppression of the Fra-1/STAT3 pathway. (**a**) Invasion of 4T1, 4T07 and EMT6 mouse breast tumor cells was detected using the Transwell assay. (**b**) Capacity of migration of 4T1, 4T07 and EMT6 mouse breast tumor cells was detected by wound healing experiments. Data are shown as mean ± s.e.m. from three independent experiments (**$P < 0.01$).

migration, invasion and metastasis. TAMs (M2-like macrophage) are able to suppress immune responses and are also capable of targeting cancer cells. The transformation from M1 to the M2 phenotype in macrophages is a critical event for tumor promotion. However, the molecular mechanisms that initiate this transformation of TAMs are still unclear. MicroRNAs are a group of small RNAs that regulate gene expression, some of which have also been reported to be oncogenes involved in tumorigenesis. Recently, certain miRNAs have been discovered that are involved in the progression of monocyte–macrophage differentiation into osteoclasts.[23–25] Nevertheless, a few studies have been conducted to investigate the role of miRNAs that control the phenotype transformation of TAMs.

To date, numerous miRNA profiling methods, such as real-time PCR, bead-based flow-cytometric profiling technology and microarray, have been identified for the diagnosis, staging, progression,

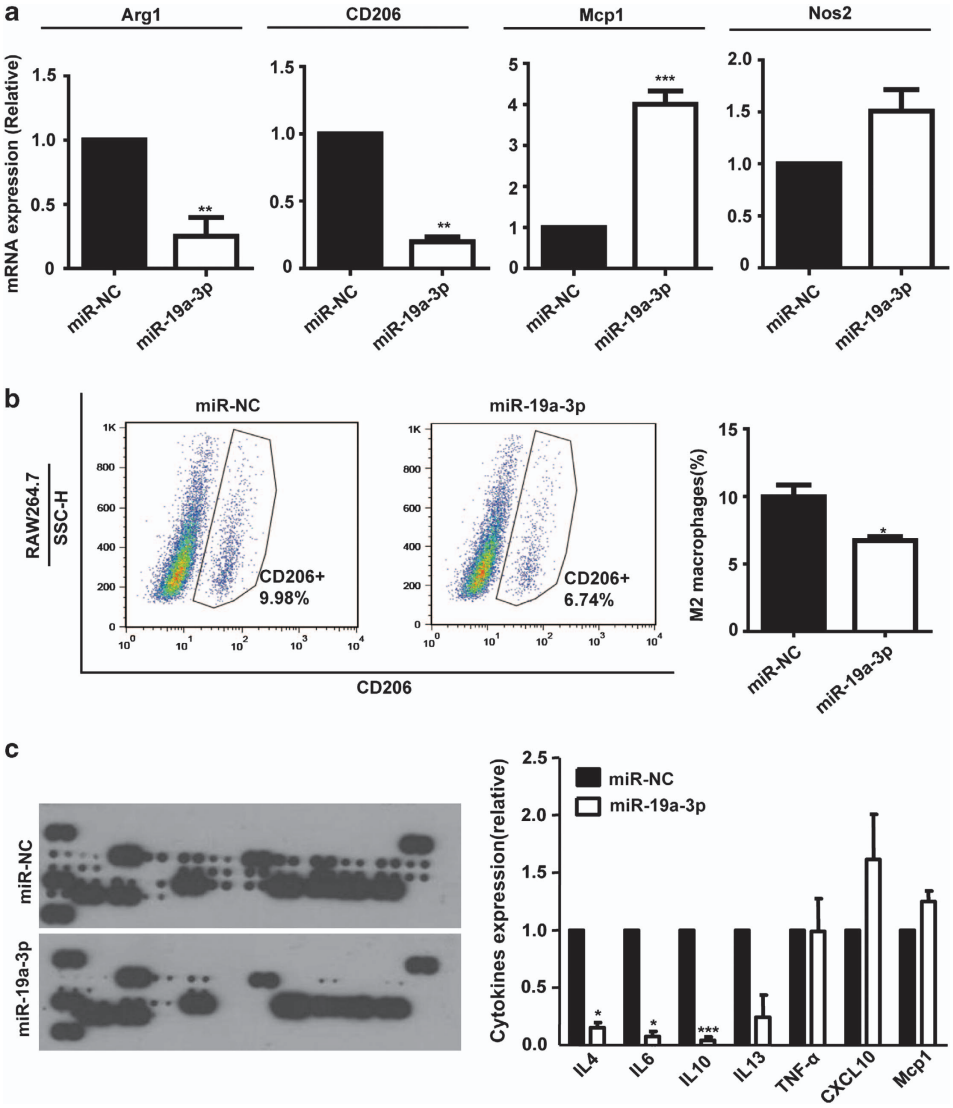

Figure 4. MiR-19a-3p downregulates the M2 phenotype of macrophages by suppression of the Fra-1/STAT3 pathway. (**a**) Arg1, CD206, Mcp1 and Nos2 expressions were detected in RAW264.7 cells transfected with the miR-19a-3p mimic and compared with a negative control. (**b**) The proportion of M2 macrophages that gated with CD206 surface markers was measured by flow cytometry ($n = 3$, $^*P < 0.05$). (**c**) Supernatants of RAW264.7 cells transfected with either the miR-19a-3p mimic or the miR-negative control were collected and the cytokine levels were detected by the mouse cytokine array panel. Data are shown as mean ± s.e.m. from two independent experiments ($^{**}P < 0.01$).

prognosis and response to treatment of human cancers.[26] However, the profiling signatures of different subpopulations of macrophages have been rarely studied. In this investigation, we combined real-time PCR and bioinformatics to explore the miRNA profiling of TAMs in both mouse and human cell lines. It was found that miRNAs such as miR-19a-3p, miR-29a and miR-503 show significant downregulation in TAMs compared with normal macrophages.

It has been widely reported that miRNAs inhibit gene expression by binding to the 3′UTR region of genes and thereby degrade the mRNA or block translation.[25,27–29] Here we demonstrated that only miR-19a-3p, and not miR-29a or miR-503, inhibits the expression of the *Fra-1* gene in TAMs. A number of studies reported that infiltration of macrophages into tumor loci correlated with poor prognosis for multiple cancer types.[30–32] Fra-1 was also reported to be a key transcription factor during the progression of transformation of TAMs. In our previous study, Fra-1 was found to act as a proto-oncogene in initiating the activation of the IL-6/JAK/STAT3 signaling pathway. Thereby, a malignant switch was created in breast tumor cells, leading to the increased release of proangiogenic factors, including matrix metallopeptidase 9 (MMP-9), VEGF and transforming growth factor beta (TGF-β), from tumor cells. This also included the intensified invasion and progression of breast cancer.[19] On the other hand, the results of this study showed that miR-19a-3p also inhibits other signaling pathways that promote M2 polarization of macrophages, especially the glucocorticoid pathway, which promotes macrophage recruitment and M2 macrophage activation by activating the STAT3 pathway[33] and the NFAT pathway, which leads to increased production of M2 cytokines such as IL-4 and IL-6 in Th2 cells.[34] Meanwhile, the estrogen pathway was reported to inhibit the alternative activation of TAMs.[32] Moreover, decreased levels of M2 cytokines secreted by TAMs were also observed. Significantly, when miR-19a-3p was overexpressed in TAMs, the facilitated capacity of the migration and invasion of breast cancer cells was attenuated to varying degrees. In this regard, it was reported that migration and invasion of colon tumor cells could be inhibited via silencing of Fra-1,

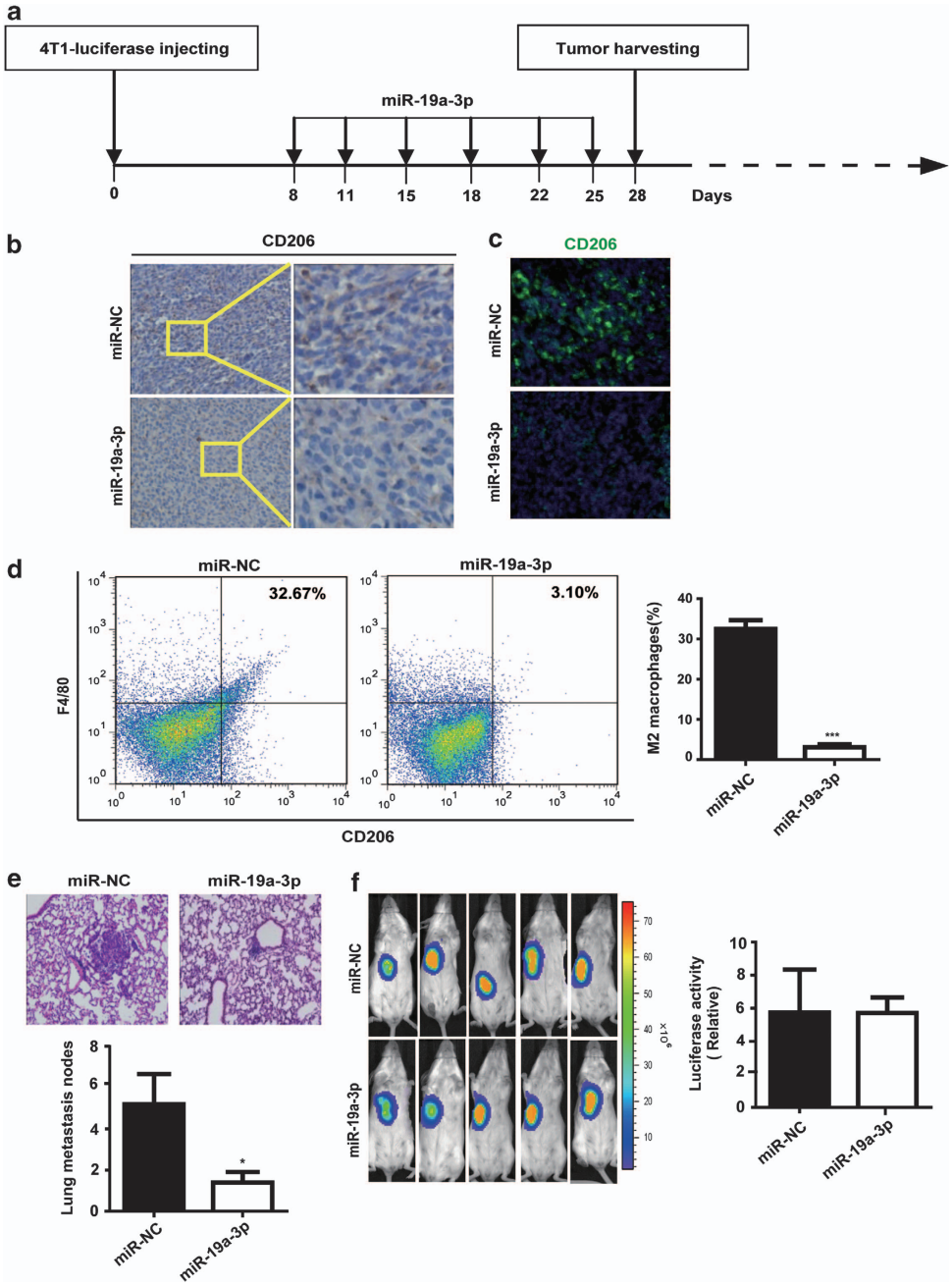

Figure 5. MiR-19a-3p inhibits breast cancer cell metastasis by regulating the M2 phenotype of TAMs in a Balb/c mouse breast tumor model. (**a**) 4T1 cells labeled with luciferase were incubated in Balb/c mice. These mice, xenografted with 4T1 cells, were injected intratumorally twice weekly with miR-19a-3p agomir or a negative control. Tumor masses and lungs from these animals were harvested after 3 weeks and CD206-positive cells were detected after injection with miR-19a-3p agomir by immunohistochemistry (**b**) and immunofluorescence staining (**c**). (**d**) F4/80 + CD206 + cells were detected in tumors injected with miR-19a-3p agomir and compared with negative control. The lung metastases of Balb/c mice ($n = 3$, $^{***}P < 0.001$) (**e**) and tumor volume (**f**) were measured after injecting miR-19a-3p agomir into the 4T1 tumor model.

which binds to the promoter of MMP-1 and MMP-9.[35–37] These results revealed that miR-19a-3p has an important role in inhibiting M2 macrophage polarization by regulating M2 polarization-related signaling pathways.

There exists abundant evidence to suggest that the proportion of TAMs within the tumor mass is correlated with the metastatic potential of the tumor. In fact, by remodeling the tumor microenvironment and relaxing the extracellular matrix of tumor cells, TAMs allow tumor cells to detach from the tumor mass and disseminate, resulting in distant metastasis. Here, in the Balb/c mouse model, we found that intratumoral injection of miR-19a-3p not only decreased the population of TAMs but also inhibited lung metastasis of 4T1 breast cancer cells. In addition, IL-6 was found to act as a negative regulator of miR-19a-3p in the tumor microenvironment. Although the detailed mechanism of IL-6 regulating miR-19a-3p remains to be explored, data reported here make it clear that the tumor microenvironment promotes the M2 phenotype of TAMs by inducing an miRNA profile that inhibits miR-19a-3p expression in order to activate the signal pathways that promote M2 polarization of TAMs.

In summary, the findings reported here support the hypothesis that miR-19a-3p, which regulates TAMs in the breast tumor

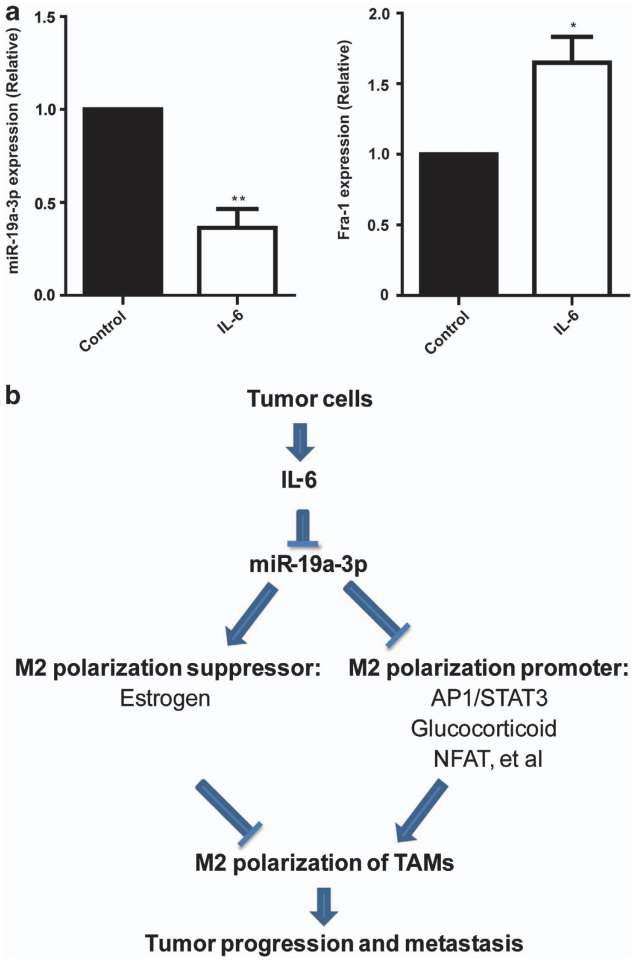

Figure 6. IL-6 activates M2 polarization-related signal pathways of TAMs by inhibiting miR-19a-3p expression. (**a**) MiR-19a-3p and Fra-1 expression were detected in RAW264.7 cells treated with IL-6 and compared with negative control. (**b**) Schematic representing M2 polarization of TAMs through microRNA-19a-3p regulation.

microenvironment, is able to regulate the phenotype of TAMs by targeting the Fra-1 gene and other genes in its downstream signaling pathway. The downregulation of miR-19a-3p expression in TAMs is likely due to TME induction, which promotes transformation of M1 to M2 and results in the enhancement of migration and invasion of breast cancer cells (Figure 6b). Therefore, it is possible that miR-19a-3p could also become a potentially useful therapeutic target for the treatment of breast cancer. In this regard, chemical modifications, some of which have already been developed for siRNAs, can be applied to miRNAs.[38] In addition, nanoparticle-mediated delivery of synthetic miRNAs, specifically to tumor-associated leukocytes, has been reported as an efficient strategy to re-program the immunological control of metastatic cancers.[39]

MATERIALS AND METHODS

Animals and cell lines

Female Balb/c mice, 6–8 weeks of age, were purchased from the Chinese Academy of Medical Sciences Institute of experimental animals. All experiments involving animals were performed according to the guidelines on laboratory animals of Nankai University and were approved by the Institute Research Ethics Committee at the Nankai University (Permit number: 10011). Mouse breast cancer cell lines 4T1 and 4T07 and the mouse leukemic macrophage cell line RAW264.7 were obtained from Dr R A Reisfeld (The Scripps Institute, La Jolla, CA, USA). The human macrophage cell line U937 and mouse breast cancer cell line EMT6 were purchased from the Fourth Military Medical University (Xi'an, China). The 4T1, U937 and RAW264.7 cell lines were cultured in 1640 medium supplemented with antibiotics (100 U/ml penicillin–0.1 mg/ml streptomycin) and 10% fetal bovine serum (FBS). Cell line 4T07 was cultured in 1640 medium with antibiotics (100 U/ml penicillin–0.1 mg/ml streptomycin) to a final concentration containing 2 mM glutamine, 1% sodium pyruvate bicarbonate, 1% HEPES and 10% FBS.

Prediction of miRNAs by real-time PCR

The miRNAs regulating the expression of Fra-1 were predicted with the web software TargetScan (http://www.targetscan.org/). Small RNA of RAW264.7 and CD11b+ cells was purified and enriched with the miRcute miRNA Isolation Kit (Tiangen, Beijing, China). MiRNAs were prolonged by *Escherichia coli* poly(A) polymerase, and reverse transcription was performed with the miRcute miRNA First-Strand cDNA Synthesis Kit and real-time PCR with the miRcute miRNA qPCR detection kit (Tiangen). Forward primers were designed on the basis of the mature sequences of mouse miR-19a-3p, miR-503 and miR-29a, and the reverse primer was provided by the miRNA real-time PCR kit (Tiangen). The forward primer sequences are as follows: miR-19a-3p: 5′-CGCTGTGCAAATCTATGCAAAACTGAAA-3′, miR-29a: 5′-CGCTAGCACCATCTGAAATCGGTTAAA-3′, miR-503: 5′-TAGCAGCGGGAACAGTACTGCAG-3′. The universal reverse primer was provided in the miRcute miRNA qPCR detection kit.

Total RNA of raw cells was isolated with TRIzol reagent (Invitrogen, Grand Island, NY, USA). Reverse transcription was performed using TransScript First-Strand cDNA Synthesis Supermix, and real-time PCR was performed with TransScript top green qPCR supermix (TransGen, Beijing, China) in triplicate.

Western blotting

Total protein from RAW264.7 cells and U937 cells was scraped from the culture plates after 24 and 48 h. Thereafter, the miRNA mimic or inhibitor was transfected into the cells and lysed in RIPA lysate buffer, and the cell lysate was incubated on ice for 30 min and centrifuged at 13 000 rpm for 10 min before the supernatant was collected. Western blotting was performed by using polyclonal primary rabbit anti-murine antibodies Fra-1 and VEGF and mouse anti-murine antibodies STAT3 or pSTAT3, and β-actin antibody as the loading control (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Flow cytometry assays

Flow cytometry was used for the detection of cytomembrane expression of F4/80 and CD206. Macrophages transfected with either miR-19a-3p mimic or miR-negative control mimic (Ribo Biotech, Guangzhou, China) were assayed according to the instructions provided by the manufacturer (BD Bioscience, San Jose, CA, USA). Briefly, macrophages were harvested after transfection and then stained with the F4/80 antibody conjugated with PE (BD Biosciences) and with the CD206 antibody conjugated with FITC (BD Biosciences), followed by dual-color flow cytometry analysis with a BD Biosciences Digital LSR II. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Dual-luciferase report system assays

The 3′UTR of Fra-1 was cloned to the psiCHECK2 vector (Promega, Madison, WI, USA). To generate the Fra-1 mutant reporter, the seed region of the Fra-1 3′UTR was mutated to remove all complementarity to nucleotides 1–8 of miR-19a-3p. HEK293T cells were co-transfected with 300 ng of firefly luciferase reporter vector and 50 nmol miR-19a-3p or miR-negative control using Lipofectamine 2000 (Invitrogen) in 24-well plates. Luciferase assays were performed 24 h after transfection, using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity and detected using the GloMax-Multi detection system (Promega).

Signal pathway assays

Signal pathway assays were performed according to the protocol provided by the Cignal Finder Reporter Array kit (Qiagen, Germantown, MD, USA). The miR-19a-3p and miR-NC mimics were co-transfected with reporter into HEK293T cells to a final concentration of 100 nM. Luciferase activity was determined 60 h after transfection.

Cytokine secreting and stimulating assays

The RAW264.7 macrophage cell-conditioned medium was collected 24 h after the transfection of miRNA mimics. Cytokines secreted by RAW264.7 cells, either transfected with miR-19a-3p mimic or miR-negative control, were detected by the Mouse Cytokine Array Panel A (R&D, Minneapolis, MN, USA) based on the Proteome Profiler Array Protocol. Cytokine array data on the developed X-ray film were quantitated by scanning the film on a transmission-mode scanner and analyzing the array image file using ImageJ software (http//:rsbweb.nih.gov/ij/).

To detect the miR-19a-3p regulator in the TME, RAW264.7 cells were cultured in complete RPMI-1640 medium mixed with mouse IL-6 (Cell Signaling Technology, Danvers, MA, USA) at a final concentration of 10 ng/ml or without mouse IL-6 as a negative control for 6 h before RNA extraction.

Cell invasion and migration assay

Transwell chambers (Costar, Cambridge, MA, USA) with 5-μm-pore polycarbonate filters were used to assess the invasion properties of tumor cells. Cells 4T1, 4T07 and EMT6 were seeded (1×10^5 cells/chamber) on top of the cylindrical chambers in the presence of RPMI-1640 medium with 1% FBS, whereas RAW264.7 cells (1×10^5 cells/well) were seeded in the cells below in RPMI-1640 medium plus 10% FBS. Cell invasion was observed 24 h after culturing at 37 °C in 5% CO_2, and after 12 h for EMT6 cells. Noninvasive cells were removed from the bottom of the chambers using cotton swabs. Invasive cells on the other surfaces of the membrane were fixed in absolute methanol and stained with 0.1% crystal violet (Invitrogen) for 5 min at room temperature and subjected to microscopic study. Cell migration was assessed by wound healing experiments. Tumor cells (4×10^5 cells/well) were seeded in 12 orifice plates in 50% RPMI-1640 medium with 1% FBS plus 50% RAW264.7 cell-conditioned medium. After 24 h the bottom of the wells were scraped using 1 ml Pipette Tips. The widths of the wound were recorded after 24 h under a microscope. The wound healing rate was quantified by detecting the percentage of width of the closed wound versus that of the original wound.

Immunohistochemistry and immunofluorescence

For immunohistochemical staining analysis, 4T1 tumor tissue paraffin sections were fixed and stained with the Rabbit immunoCruz staining system (Santa Cruz Biotechnology) using rabbit anti-murine CD206 mAbs. Horseradish peroxidase-conjugated goat anti-mouse secondary Ab was used and slides were mounted with the cells to be visualized microscopically. For immunofluorescence, rat anti-murine CD206 mAb was used for staining of 4T1 tumor tissue frozen sections. Rabbit anti-murine Fra-1, VEGF, and mouse anti-murine Stat-3, pSTAT3, mAb were used for staining of RAW264.7 cells.

Animal experiments

The 4T1-luciferase breast cancer cells (5×10^4) were injected subcutaneously into the second mammary fat pad of each female Balb/c mouse. The miR-19a-3p agomir (chemically modified miRNA mimics) and negative control were injected intratumorally twice a week and 7 days later for 3 weeks. Luciferase activity was detected every 7 days for 3 weeks. On day 28, the tumor of each mouse was harvested for flow cytometry, immunofluorescence and immunohistochemical analyses.

Statistical analysis

Values were expressed as means ± s.e.m. Significance was determined using the Student's *t*-test. A value of $P < 0.05$ was used as the criterion for statistical significance. *Indicates significant difference with $P < 0.05$; **indicates significant difference with $P < 0.01$; ***indicates significant difference with $P < 0.001$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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